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NAME OF AUTHOR/*NOM DE L'AUTEUR* MILOCHIK, J. ANNE

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NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* J. S. CHARNOCK, PH.D.

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LIPID MODULATION OF THE ACYL PHOSPHATASE OF $(Na^{+}+K^{+})$-ATPase

BY

© J. ANNE MILOCHIK

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Lipid Modulation of the Acyl-Phosphatase of $(Na^+ + K^+)$-ATPase", submitted by J. Anne Milochik in partial fulfilment of the requirements for the degree of Master of Science.

John S. Charnock
Supervisor

Peter G. Barton

George B. Frank

Date August 9, 1978

Verily it is well for the world that it sees only the beauty of the completed work and not its origins nor the conditions whence it sprang; since knowledge of the artists's inspiration might often but confuse and alarm and so prevent the full effect of its excellence.

Thomas Mann, 1912

"Death in Venice"

ABSTRACT

The effect of membrane lipids on ($Na^+ + K^+$)-ATPase activity and on the partial reaction, the K^+-acyl phosphatase activity, was examined by comparing the values of three parameters of these enzyme activities before and after various lipid treatments. The investigation sought to answer two questions pertaining to the lipid modulation of the K^+-acyl phosphatase activity in particular: 1. Is there a requirement for a specific type of lipid? and 2. Do these lipids have a specific function?

A microsomal preparation containing ($Na^+ + K^+$)-ATPase was obtained from bovine cerebral cortex and subsequently treated with deoxycholate, (DOC), phospholipase A (PPLA), and phospholipase C (PPLC) under various conditions. The lipid depleted preparations were compared to the untreated preparations with respect to specific activity, ouabain inhibition, and temperature dependence of both the ($Na^+ + K^+$)-ATPase and of the K^+-acyl phosphatase.

Extraction with DOC enhanced both enzyme activities, whereas lipolysis with PPLA, PPLC, or both (PPLA + PPLC) lowered the activities by various amounts depending on the treatment conditions. Generally, the ($Na^+ + K^+$)-ATPase and K^+-acyl phosphatase activities were not lowered to the same extent, except in the case of PPLA treatment of a DOC-treated preparation. Ouabain inhibition was not significantly altered by any lipid treatment. However, PPLC treatment appeared to remove an inherent impediment to ouabain binding. The temperature dependence of ($Na^+ + K^+$)-ATPase activity was not altered after DOC treatment or after PPLC treatment of an untreated enzyme preparation. This was reflected by a non-linear Arrhenius plot, similar to that obtained with an

untreated enzyme preparation. However, the temperature dependence of ($Na^+ + K^+$)-ATPase was altered by either PPLA or PPLC treatment of DOC pretreated preparations, now demonstrated by a linear Arrhenius plot obtained with these preparations. By contrast, the Arrhenius plots of K^+-acyl phosphatase activities remained non-linear after similar treatments. These plots could be described by two intersecting straight lines, except in the case of PPLC-treatment of an untreated preparation or PPLC treatment of a DOC treated preparation in the presence of EDTA. In the latter case, the Arrhenius plots were neither single straight lines nor the non-linear form discussed above, but could best be described as curvilinear.

These findings suggest that the lipid requirement of the partial reaction K^+-acyl phosphatase reaction differs from that of the overall ($Na^+ + K^+$)-ATPase. The K^+-acyl phosphatase activity appeared to be less dependent on the membrane lipids than the overall ($Na^+ + K^+$)-ATPase activity. The results of this investigation could not identify the specific lipids which modulated the enzyme activities. However, a function could be proposed for the lipids associated with the enzyme reactions. A model of lipid modulation of the partial reactions composing the overall ($Na^+ + K^+$)-ATPase reaction is presented which can accommodate the proposal that the partial phosphatase activity is a portion of the ($Na^+ + K^+$)-ATPase complex and not a separate enzyme.

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INTRODUCTION

A. Adenosine Triphosphatase

The active transport of Na^+ ions was first suggested to involve the hydrolysis of energy-rich phosphate esters by P.C. Caldwell in 1956 (26). This suggestion led Skou (132) to investigate the active extrusion of Na^+ ions from the legs of the shore crab, *Carcinus maenas,* in the following year. Because the hydrolysis of ATP released large amounts of energy, Skou proposed that the membrane bound enzyme which hydrolyzed this phosphate ester in the presence of both Na^+ and K^+ was responsible for Na^+ transport, also. This enzyme, $(Na^+ + K^+)$-activated-ATPase (E.C.3.6.1.3.), has since been found to hydrolyze not the ester bond of ATP, but the terminal acid anhydride bond thereby releasing a larger amount of energy than hydrolysis of the ester bond would have released. $(Na^+ + K^+)$-ATPase is found in many tissues especially those whose physiological function depends on the regulation of ion concentrations across cell membranes, e.g., nervous tissue, (especially brain) (3, 31, 47, 56, 64, 132, 140, 144), mammalian kidney cortical cells (24, 27, 28), turtle bladder mucosal cells (129), rectal glands of fish (62, 63), avian salt glands (44, 45), gastric mucosal cells (136), parotid glands (139), blood cells (11, 31, 85, 99, 152, 160) and heart tissue (7, 25, 158). The role of ATPase in regulating ion transport made it an essential enzyme in the evolution of the animal kingdom from its aquatic environment to the land and to the air.

The $(Na^+ + K^+)$-ATPase possesses characteristics consistent with a physiological role in the ion transport system as the "Sodium Pump":

1. it is located close to the nerve, presumably in the axon sheath, and;
2. its activity increases after an action potential when the intra-axonal Na^+ concentration becomes elevated.

Its activity is dependent on Mg^{++} ions, as well as on Na^+ and K^+ ions, and it can be inhibited by Ca^{++} ions (51).

Recently, Hokin *et al.* (61, 62, 63), Schwartz *et al.* (127) and Racker *et al.* (100, 101), presented evidence that a directional transport function could be introduced into lipid vesicles by including a highly purified (Na^+ + K^+)-ATPase protein. These experiments confirmed that this enzyme is both the site of energy transduction and of ion translocation in cell membranes. Their work with liposomes (lipid vesicles) emphasized two important features of the (Na^+ + K^+)-ATPase which are mandatory for its function as the "Sodium Pump", namely,

1. the transmembrane localization, which permits the enzyme to act as a vehicle for translocating ions across the membrane, and;
2. the asymmetric activation of Na^+ and K^+ ions. In most investigations, the (Na^+ + K^+)-ATPase was orientated in the liposomes inside-out to the natural way, but this still confirmed the asymmetry of its activation (63).

The present level of understanding of (Na^+ + K^+)-ATPase function owes much to the early work of Schatzmann (124) and Repke (109, 110, 111). Both investigators studied the enzyme function by using specific inhibitors, the cardiac glycosides. In 1953, some five years before Skou originally isolated the (Na^+ + K^+)-ATPase enzyme, Schatzmann (124) found that the cardiac glycoside, digitalis, inhibited the active transport of Na^+ and of K^+ ions across the cell membrane of erythrocytes. Skou then found that g-strophanthin (ouabain), (Fig. 1) a cardiac glycoside which is more water soluble than digitalis, inhibited the activating effect of Na^+ and K^+ on a crab ATPase preparation, presumably by interfering with the binding of the cations to the enzyme (132). The sensitivity of the ATPase to cardiac glycoside inhibition in various tissues in different species was subsequently studied. The difference in topography of the external membrane surface surrounding the K^+ site of the $(Na^+ + K^+)$-ATPase was claimed to be responsible for different properties of cardiac glycoside inhibition in various species. Repke and Portius (111), in 1965, and Erdman (42), in 1973, discerned various kinetic and thermodynamic properties of digitalis and ouabain binding to the enzyme.

As early as 1960, Repke and Portius (98, 111) decided that some peculiar features of digitalis pharmacodynamics could not be understood from either the distribution of the drug in the whole animal or from the metabolic alterations which followed. They suggested that those features could depend on the properties of a hypothetical digitalis receptor in the tissues. In 1965, they chose to investigate the $(Na^+ + K^+)$-ATPase enzyme as a possible receptor of cardiac glycosides in cardiac muscle, as did Skou in crab nerve preparations. Repke and Portius found that the locus of action of digitalis was the membrane fraction where $(Na^+ + K^+)$-ATPase activity was found, and that the mode of action was the inhibition of $(Na^+ + K^+)$-ATPase. They also found that inhibition by digitalis could be antagonized by K^+ ions, and by a high concentration of Ca^{++} ions, but inhibition was enhanced by Na^+ ions, and by a low concentration of Ca^{++} in the presence of K^+. The optimal pH for activity was found to be between 7 and 8 (17). Inhibition by cardiac glycosides was found to be less efficient during hypothermic states in hibernating animals (58). This result suggested that the inhibitory action of cardiac glycosides was sensitive to temperature. Repke (111) noted that the heart muscle displayed a peculiar sensitivity to the cardiac glycosides, although it was not the organ of the body which possessed the highest affinity for these drugs. This suggested to him that the functional significance of an inhibition of transport-ATPase varied in each organ.

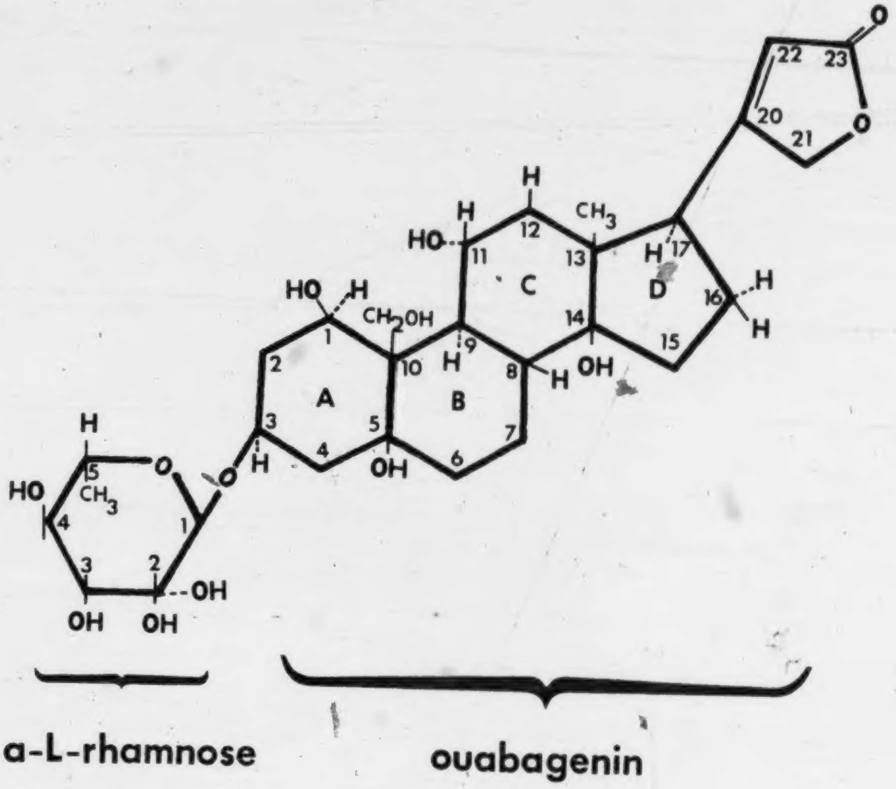


Fig. 1. Structure of ouabain (from Sim, 1967 (130)).

Erdmann *et al.* (42, 43) also investigated the temperature and lipid dependence of cardiac glycoside binding. Both Repke *et al.* (98) and Erdmann *et al.* (42) investigated the relationships of the structure of these drugs to their ability to bind to the ATPase. Besides investigating the cardiotonic steroids, Portius and Repke (98) also investigated some steroids of the cardenolide and bufandienolide series, the Veratrum alkaloids, hormones, and Erythrophleum alkaloids. They suggested that the important structural requirements for drug binding were:

1. a carbonyl group associated with a carbon-carbon double bond on the side chain;
2. the steroid or perhydrophenanthrene nucleus itself (referred to as the "fixing" group);
3. the sugar component which is a supporting group whose polar nature can counteract a non-specific binding to other proteins and can facilitate a more complete fixation at the active centre.

In 1974, Erdmann *et al.* (43) suggested that this enzyme "may contain" the cardiac glycoside receptor. They carried out binding studies based on this theory, and concluded as did Repke *et al.* (98), that the structures on the molecule which were necessary for binding and, hence, inhibition were:

1. the unsaturated lactone group;
2. the steroid nucleus with a cis configuration of the A:B ring junction; and,
3. the sugar component.

The exposition of the reaction mechanism of ATP hydrolysis by $(Na^+ + K^+)$-ATPase whereby the transport of ions occurred, challenged the biochemical investigators of the 1960's. Using the classical approach of measuring the degradation of radioactively labelled substrate, the following reaction sequence for ATP hydrolysis and ion transport by $(Na^+ + K^+)$-ATPase was eventually elucidated (4, 40, 133):

1. $E + ATP \xrightleftharpoons{Mg^{++},\ Na^+} E \sim P + ADP$

2. $E_1 \sim P \xrightleftharpoons{Mg^{++}} E_2 \sim P$

3. $E_2 \sim P \xrightleftharpoons{K^+} E_2 + P_i$

4. $E_2 \longrightarrow E$

In the first step, a kinase reaction, the enzyme "E" cleaves the terminal phosphate from ATP, becomes phosphorylated and generates the product ADP. This reaction requires Mg^{++} and Na^+ for activation, and it is believed that Mg^{++} - ATP is the real substrate for this reaction (37). In the second step, the phosphorylated enzyme undergoes a conformational change represented as $E_1 \sim P$ to $E_2 \sim P$. (The properties of these two forms of the enzyme have been shown to be different (90, 97). In the third step, the phosphatase reaction, the phosphorylated enzyme is dephosphorylated under the influence of K^+ ions. This particular step is the one most readily inhibited by cardiac glycosides (28, 29). In the fourth step, the enzyme E_2 is spontaneously reconverted to the E_1 form and the cycle of reactions is complete.

Because the phosphatase activity completes the mechanism initiated by the kinase activity of the first step, and because it is specifically inhibited by cardiac glycosides, it is of special interest pharmacologically. The greater understanding of the regulation of this partial reaction by ions and lipids is expected to lead to a greater understanding of the mechanism of action of the pharmacologically active cardiac glycosides, which in turn may effect their more rational clinical use. It is for these reasons that this reaction has been studied so intensively and why it is the central topic of this thesis. Of specific concern is the lipid modulation of the phosphatase reaction, especially as it pertains to the cardiac glycoside inhibition of the reaction. However, before discussing the influence of the membrane lipids on the overall and partial reactions of ($Na^+ + K^+$)-ATPase, other properties of the phosphatase reaction will first be discussed.

B. The Phosphatase Reaction

The phosphatase activity of ($Na^+ + K^+$)-ATPase was first described by Judah *et al.* (70) in 1962 as being stimulated by K^+, inhibited by ouabain, and capable of hydrolyzing the substrate *para*-nitrophenylphosphate (*p*-NPP). Because several relatively non-specific acid and alkaline-phosphatases exist in the cell membrane, in this thesis, the term "phosphatase reaction" will be used to specify the third step of the ($Na^+ + K^+$)-ATPase reaction sequence described above. The natural substrate for this reaction is the phosphorylated enzyme intermediate $E_2 \sim P$ which is known to be an acyl - phosphate (70, 123, 165).

During the initial stages of studying the phosphatase reaction, investigators proposed that the phosphatase enzyme was physically separate from the ($Na^+ + K^+$)-ATPase complex, although both activities were found simultaneously in membrane preparations (3, 13, 37, 74, 95). After more than a decade of active research, current opinions favour the theory that the phosphatase is an integral part of the ATPase complex and not a distinct enzyme; but evidence is still far from unequivocal.

Several properties of the phosphatase have been examined in greater detail in attempts to answer this particular question. These properties include:

1. the localization of phosphatase activity in cell membranes;
2. the substrate specificity;
3. the ion dependence and its relationship to the transport of Na^+ and K^+, and to the regulatory sites for ions, nucleotides, substrates and inhibitors;
4. the inhibitors of the phosphatase activity and their mechanism of action; and,
5. the lipid modulation of the reaction at the active site(s), at the ion regulatory and substrate binding sites, and at the inhibitory site(s).

These various subtopics of the phosphatase reaction will now be elaborated upon individually, necessarily with comparison to the corresponding properties of the overall $(Na^+ + K^+)$-ATPase reaction.

1. Localization

Before considering other properties of the $(Na^+ + K^+)$-ATPase and phosphatase activities, their location in the cell must be described. $(Na^+ + K^+)$-ATPase is responsible for transporting Na^+ and K^+ ions into and out of the cell thereby maintaining the intra- and extracellular ionic balance; therefore it must necessarily be located in the plasma membrane. However, its presence in intracellular membranes is also well documented (3, 92, 163).

Albers (3) investigated the subcellular distribution of $(Na^+ + K^+)$-ATPase and K^+-phosphatase activities in rat brain tissue. They found the major amounts and the highest specific activities of both enzyme activities in a crude mitochondrial fraction, whereas other investigators found the $(Na^+ + K^+)$-ATPase to be localized in microsomal fractions of tissue homogenates, or in a nuclear fraction containing cell debris and intact cells (163). The distributions of the two enzymes differed in the mitochondrial subfractions. The $(Na^+ + K^+)$-ATPase was found in the largest proportion and highest concentration in a subfraction which also contained cholinergic nerve endings. In general, the distribution of $(Na^+ + K^+)$-ATPase resembled that of acetylcholinesterase (except that a lower concentration of $(Na^+ + K^+)$-ATPase was found in the microsomes). In contrast, the K^+-activated phosphatase was most highly concentrated in the subfraction containing noncholinergic nerve endings. Both enzymes were tightly bound to the membranes, as shown by the retention of activity after osmotic shock and by the insignificant activity in synaptic vesicles and axoplasm. These results indicated to Albers (3) that the $(Na^+ + K^+)$-ATPase was closely associated with membranous components of cell fractions containing nerve endings; that it was distinct from the K^+-phosphatase, and that it exhibited a different structural association.

On the other hand phosphatase activity has usually been localized wherever the $(Na^+ + K^+)$-ATPase activity has been demonstrated, although Nagai and Yoshida (91) have reported that the intracellular distributions of the activities in the guinea pig brain were not identical. Nagai, Izumi and Yoshida (90) found that phosphatase activity was about 10% of the value of $(Na^+ + K^+)$-ATPase activity in guinea pig brain, and that this distribution remained relatively constant even after treatments of the membrane preparations with NaI, protamine, *p*-chloromercuribenzoate (PCMB), nigrosin and acetone.

Ernst (44, 45) carried out a histochemical investigation of avian salt gland to locate the phosphatase enzyme. Essential to the procedure was the assumption that the K^+-phosphatase activity was an integral part of the enzyme transport system, and manifested the terminal dephosphorylation step of the $(Na^+ + K^+)$-ATPase reaction, i.e., accepting the

the proposed reaction mechanism cited on P7 and rejecting of Albers' conclusions. The procedure depended on the conversion of $Sr_3(PO_4)_2$ to $Pb_3(PO_4)_2$ which could then be visualized in the electron microscope. The substrate used to demonstrate phosphatase activity was *p*-nitrophenyl phosphate (*p*-NPP) which was enzymatically hydrolyzed to *p*-nitrophenol (*p*-NP). Ernst found that the products *p*-NP and Pi were localized on the cytoplasmic side of the secretory cell lateral and basal plasma membranes, and that little product was seen on the apical surface of the secretory cells or on the endothelial surfaces of the capillaries. The *p*-NPPase activity on the cytoplasmic side of the basal and lateral plasma membranes was ouabain-sensitive and K^+- and Mg^{++}-dependent. Artifactually produced *p*-NP precipitates were larger and more numerous and could be distinguished from enzymatically produced precipitates. The precipitates produced by non-mitochondrial enzyme activity were located intracellularly at some distance from the plasma membrane. These studies supported the data from erythrocyte studies which suggested that a single enzyme was responsible for the K^+-dependent and K^+-independent phosphatase activities (49).

Ernst (45) cautioned that cytochemical data must be balidated by exhaustive controls and supportive biochemical and physiological data. Few other studies have been performed on the cytochemical localization of $(Na^+ + K^+)$-ATPase because of the difficulties in interpreting the results (8, 31, 46). Most methods have depended on the visualization of enzymatically produced lead phosphate deposits (85, 89). Very recently, Terva (149) tried to localize *p*-NPPase activity as well as ATPase activity in rat retinal endothelium and encountered problems with fixation procedures.

2. Asymmetry

The side of the cell membrane where the phosphatase reaction occurs has been investigated. Because the initial kinase reaction occurs on the internal surface of the cell membrane, the dephosphorylation also would be expected to occur on the internal surface. K^+ might stimulate the phosphatase reaction from the external surface of the membrane and, in the course of the overall reaction, be transported inwards .Since the cardiac glycosides have not been shown to penetrate the cell membrane they might also inhibit on the same side as K^+ stimulates.

Rega *et al.* (104, 105) investigated the phosphatase reaction in red cell ghosts (a normal erythrocyte which has had its haemoglobin removed by treatment in a hypotonic medium). They found that the phosphatase enzyme required the substrate to be present internally and the activator (K^+) to be present externally. In 1972 (53), they also found that the substrate *p*-NPP inhibited cation movements generated by the overall (Na^+ + K^+)-ATPase activity, allegedly by combining with the active site of the membrane phosphatase on the inner surface of the cell membrane. The degree of inhibition depended on the intracellular concentration of ATP.

Askari *et al.* (11, 14) similarly claimed that the membrane-bound phosphatase of the erythrocyte was oriented towards the inside of the cell, but in disagreement with the reports of Rega *et al.* (104, 105), they claimed that it was supplied with substrate and nucleotide from the outside. They found that *p*-NPP was hydrolyzed when added to the medium outside the erythrocyte, and that the product *p*-NP did not accumulate significantly on the inside of the red cells or ghosts. However, *p*-NP is a weak acid of pKa 7.15, so that at the pH used in Askari's experiments it could pass through the membrane regardless of the side on which it was formed. Modifiers of phosphatase activity, e.g., K^+, Na^+, ATP, ouabain, orthophosphate and oligomycin demonstrated weak effects and limited permeability at very low concentrations in the external medium. However, it is possible that these modifiers could act on the outside while the active site of dephosphorylation could be oriented toward the inside. In this sense, the phosphatase reaction could be considered "transmembrane" as is the overall $(Na^+ + K^+)$-ATPase reaction.

3. The Phosphorylated Intermediate

The natural substrate of the phosphatase reaction under study is the phosphorylated enzyme designated $E \sim P$ (17, 29). After formation by the hydrolysis of the terminal phosphate of ATP, the phospho-enzyme complex spontaneously undergoes a conformational change represented as $E_1 \sim P \longrightarrow E_2 \sim P$, and is then dephosphorylated from the $E_2 \sim P$ form in a K^+-dependent reaction (86). There is no separate or parallel reaction to dephosphorylate the $E_1 \sim P$ form.

Artificial substrates which are satisfactory for studying the acylphosphatase enzyme include carbamyl phosphate, acetyl phosphate and *p*-nitrophenyl phosphate (listed in decreasing order of Km values) (68, 90, 91). Umbelliferone phosphate is particularly suitable because its fluorescent product, umbelliferone, can be easily quantitated even when small amounts are produced (95). The presence of ATP as a competitive substrate was found to inhibit the K^+-acyl phosphatase reaction (67). Because *o*-phosphoryl serine or α- or β-glyceryl phosphate were not hydrolyzed, Yoshida (163) suggested that the acyl-phosphate intermediate does not have a phosphoryl-serine linkage. The E ∿ P intermediate isolated by Shamoo (121, 122) in 1970 was an acid stable, energy-rich glutamyl-γ-phosphate.

The hydrolysis of the E ∿ P intermediate and of artificial substrates by acetyl phosphatase is stimulated by K^+, while the hydrolysis of ATP by (Na^+ + K^+)-ATPase is a Na^+-dependent step. The hydrolysis of artificial substrates also differs in not requiring the initial binding of the phosphate to the enzyme (68). Askari and Koyal (11) did not totally accept this postulate and proposed that a binding site existed in the red cell ghost which was different from the ATP binding site leading to the E_1 ∿ P formation. They suggested that an acetyl phosphate could bind directly at this hydrolytic site to directly form an E ∿ P intermediate, most likely the E_2 ∿ P form. However, they also suggested that the conformational change itself, which provided a favourable substrate for K^+-stimulated hydrolysis was stimulated by the binding of a nucleoside triphosphate in the presence of Na^+ and Mg^{++} and was not stimulated by the formation of an E ∿ P complex *per se*.

Robinson (114, 115) also proposed that two reaction sites co-existed on the (Na^+ + K^+)-ATPase enzyme: one where binding of nucleotide substrate activated phosphorylation and, in turn, activated a phosphatase reaction; and another site which would be identical to the hydrolytic site of the phosphatase reaction of an E $\sim$ P intermediate and would be sensitive to the initial site of phosphorylation. He thus proposed an $E_3 \sim P$ conformation for the hydrolytic site in a reaction scheme illustrated in Fig. 2, Page 16. In Robinson's reaction scheme, ATP phosphorylated a glutamyl residue thereby activating the enzyme; the phosphate group was then transferred in a K^+-dependent step to the distinct hydrolytic site, perhaps as a succeeding phosphate was donated by another ATP to the glutamyl residue. Direct access to the hydrolytic site was available to the acetylphosphates in a K^+-activated step. When Na^+ and K^+ were in the medium, he suggested that the ATPase pathway was followed. These experiments suggested that ATP failed to undergo significant K^+-activated hydrolysis. In addition the hydrolyzed terminal phosphate failed to bind to the enzyme. This behaviour demonstrated that the ATP molecule obtained direct access to the terminal hydrolytic site only with great difficulty. This model appeared to be the most compatible with experimental evidence. However, a distinct, third hydrolytic site would not necessarily be required to explain all the observed phenomana.

The models proposing two binding sites and those proposing the competitive inhibition of ATP and acetyl phosphate substrates seem to favour the theory that the K^+-phosphatase activity is part of the same enzyme macromolecule as $(Na^+ + K^+)$-ATPase or part of the same enzyme complex. Israel and Titus (67), and Sachs *et al.* (123) proposed a slightly more conservative hypothesis in which the K^+-acetyl phosphatase which hydrolyzed artificial substrates was distinct from the K^+-dependent activity which hydrolyzed the natural substrate, the phosphorylated intermediate of the $(Na^+ + K^+)$-ATPase. (Robinson's proposal, on the other hand, invoked the same activity for different substrates.) The pathway proposed by Israel and Titus for enzyme action on artificial substrates is illustrated in

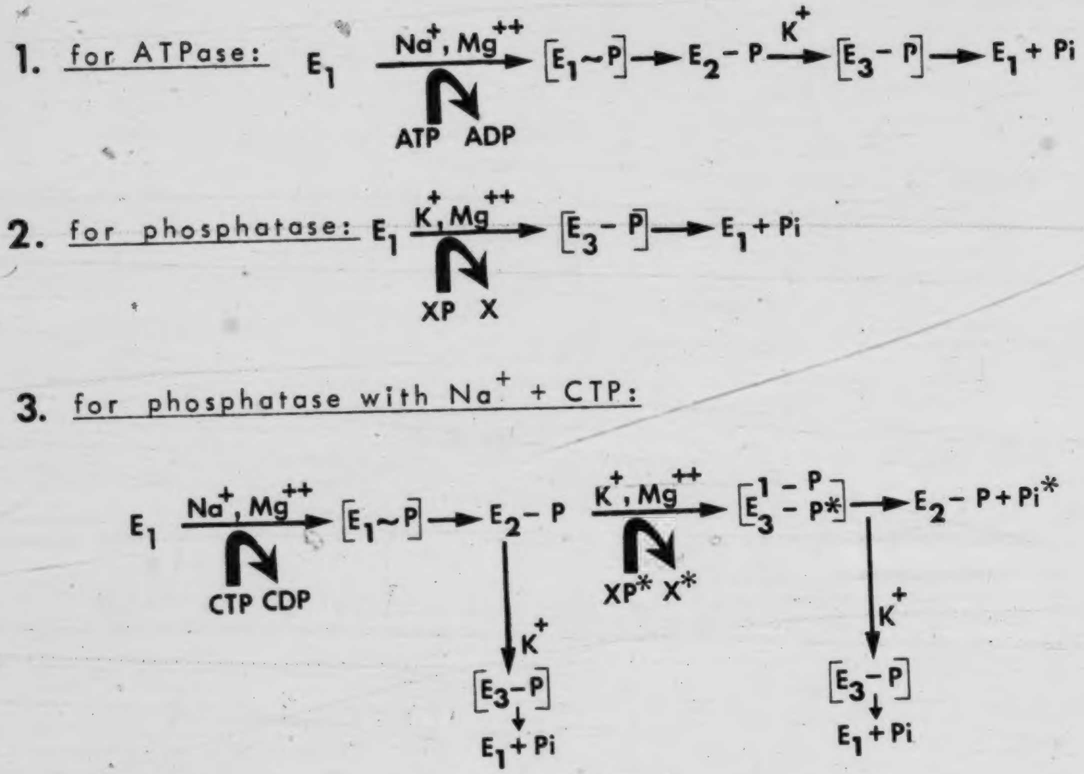


Fig. 2. Reaction scheme of ATP hydrolysis (according to Robinson, (1970) . Full details are given in reference #116.)

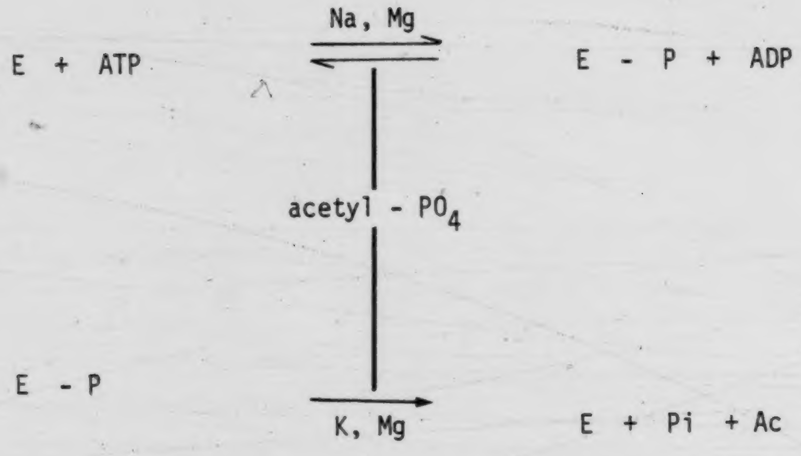


Fig. 3. Pathway of hydrolysis of phosphates (according to Israel and Titus (1967). Full details are given in reference 67.)

Fig. 3, Page 17.

4. Ions and Ion Transport

The comparison of the influences of the ions on the overall and partial reactions of ($Na^+ + K^+$)-ATPase provide more heat than light to the argument of whether these activities arise from one enzyme or two.

(a) Potassium ions

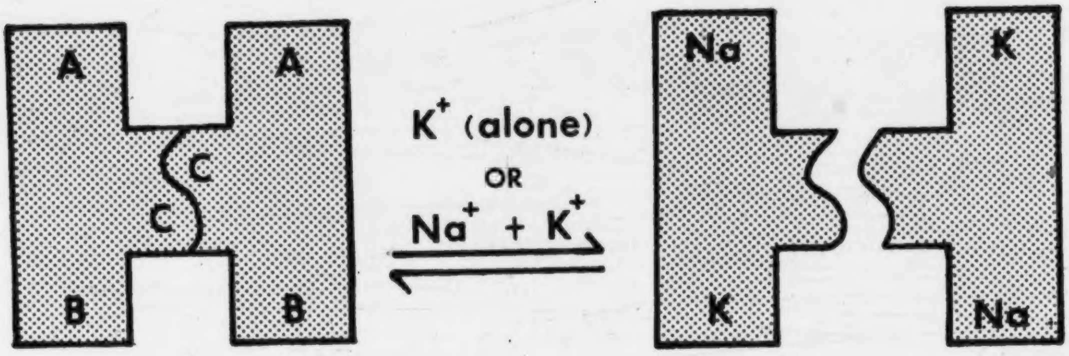
The K^+-activated phosphatase reaction does not have an absolute requirement for K^+, but can be stimulated also by Rb^+, Cs^+, NH^+_4, or Li^+, but not by Na^+ (listed in decreasing order of affinity (17, 24)). The active transport of Na^+ in the overall ($Na^+ + K^+$)-ATPase reaction could be stimulated by the above ions in the reverse order of efficacy (24). This finding might suggest that the phosphatase enzyme is an integral part of the ($Na^+ + K^+$)-ATPase complex. (In contrast, the activation of the initial kinase reaction of the ($Na^+ + K^+$)-ATPase sequence has an absolute requirement for Na^+ (74)). Li^+ can replace K^+ in the ($Na^+ + K^+$)-ATPase reaction, but not in the phosphatase reaction unless CTP or an acetyl phosphate is present with Na^+ (91, 115). These diverse characteristics suggested that in the red blood cell two separate enzymes are involved in cation transport. But Albers *et al.* (4) suggested that these enzymes were identical because of similarities in K^+ ion and pH requirements of K^+-phosphatase and ($Na^+ + K^+$)-ATPase of microsomal fractions of Electrophorus electric organ.

The interaction of K^+ and Na^+ in the overall ($Na^+ + K^+$)-ATPase reaction was said to be one of "synergistic activation" (5). K^+-activation without Na^+ involved two different sites: one which regulated substrate accessibility, another which increased catalytic potential. Regulatory effects of Na^+

and K^+ were defined as those which lowered the half-maximal substrate concentration of the enzyme reaction; and catalytic effects of K^+ were defined as those which influenced the velocity of the reaction. Without Na^+, the high affinity of the (Na^+ + K^+)-ATPase was absent. With Na^+, catalytic sites were "opened" and the K^+ affinity rose above that possible with K^+ regulatory sites alone. More high affinity catalytic sites became available as more K^+ regulatory sites were occupied by K^+ instead of by Na^+.

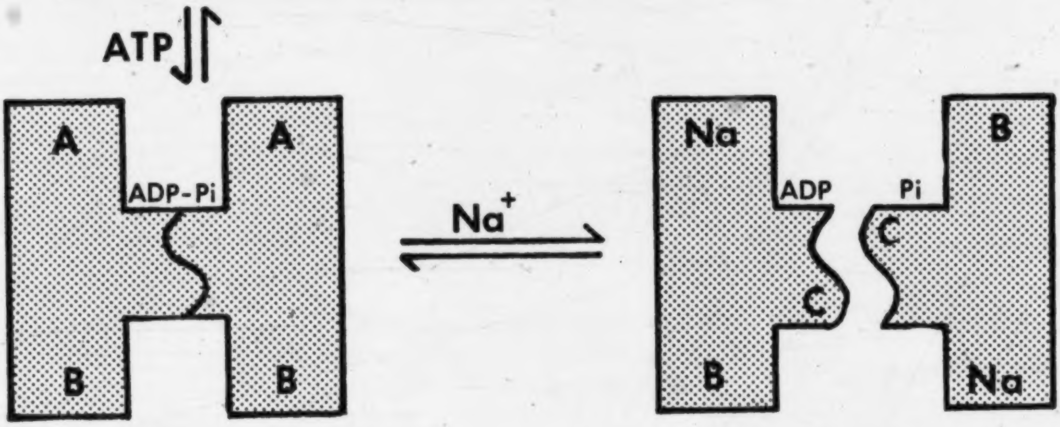
A mathematical model devised by Albers (6) via a Tiepel-Kochland analysis is consistent with the subunit dissociation scheme described above in which the catalytic sites are "unmasked" by either Na^+ or K^+ acting at separate regulatory sites. Swann and Albers (140) extended this scheme to construct a "Dimer Relaxation Model" of ion influence on (Na^+ + K^+)-ATPase enzyme action as described in Fig. 4, Page 20. In this model, both ATP-binding and ATP-dependent phosphorylation determined the conformational transitions of the active transport units. ATP could compete with *p*-NPP at the phosphate catalytic site especially when saturating concentrations of both Na^+ and K^+ were used, in which case, the effects of K^+ prevailed.

Robinson (112) also proposed a model for cooperative interactions of Na^+ and K^+. He suggested that a fraction of the K^+ sites (not identical to those which mediate a response) could be competitively occupied by Na^+, thereby being converted to Na^+ inhibitory sites. However, the slight activation of phosphatase by Na^+ which can be inhibited by K^+ is difficult to reconcile with ion competition for the same site.



T conformation inactive as a phosphatase but can phosphorylate itself.

R-relaxed form - an active phosphatase. The catalytic sites are exposed when appropriate amounts of sodium and/or potassium bind to the regulatory sites.



T conformation is stabilized by ATP-phosphorylation makes the "T" form unstable with respect to the "R" form.

The electrostatic repulsion between the enzyme acyl-phosphate and the enzyme bound ADP could promote the transition from the T to the R conformation. Only the "dephospho-R" form can hydrolyze pNPP.

Fig. 4. Dimer Relaxation Model of cation binding to (Na^+ + K^+)-ATPase (140). (Taken from Swann and Albers (1975). Full details are presented in reference no. 140.)

K^+ effects on artificial substrates have also been examined. Garrahan *et al.* (50) found that the K^+-coupled rate of hydrolysis of a substrate equalled the total rate of hydrolysis at various K^+ concentrations, and concluded that a single enzyme was responsible for the hydrolysis of substrate in the presence and absence of K^+. K^+ lowered the Km for the phosphatase substrate and enhanced the Vmax; both values tended toward a limiting value as the concentration of K^+ was increased (106). Robinson (115) described K^+ and *p*-NPP as being heterotropic modifiers of each other with each affecting the other's Km in the *p*-NPPase reaction.

(b) Sodium ions

The effects of Na^+ on the phosphatase reaction have already been alluded to when considering its competition with K^+. Very simply, Na^+ inhibits the phosphatase reaction by competing with K^+, possibly for the same binding sites. However, in the natural environment, Na^+ is necessarily present to activate the kinase reaction which yields the phosphorylated enzyme, the substrate for the phosphatase reaction. While these reactions are occuring, these ions are also being translocated from one side of the membrane to the other against their concentration gradients. Unlike K^+, the requirement for Na^+ in the $(Na^+ + K^+)$-ATPase reaction is absolute (74).

In the presence of even a small amount of ATP, Na^+ indirectly activates the K^+-phosphatase. The Na^+-dependent binding of ATP to the enzyme complex, rather than the phosphorylation of the enzyme by ATP, was proposed to be the primary event in the modification of the enzyme to lower the Km value for K^+. The enhanced affinity for K^+ leads to the activation of the step which hydrolyzes the phosphorylated enzyme intermediate. This hydrolysis in turn leads to the conformational change of the enzyme in the membrane to cause the translocation of Na^+ and, hence, of K^+ across the membrane (10, 165).

The phosphorylation of the enzyme by ATP provides the energy necessary for active transport. Co-existing Na$^+$ and K$^+$ sites might permit separate, but interdependent, channels for cation movement (112).

The enhanced selectivity of the (Na$^+$ + K$^+$)-ATPase enzyme for K$^+$ after Na$^+$ combined at K$^+$ sites (in the presence of ATP) could also be explained in terms of "catalytic" and "regulatory" sites for K$^+$ ions. The K$^+$ regulatory sites can be occupied by Na$^+$, thereby activating the enzyme. In the activated state, the enzyme and K$^+$ regulatory sites are more highly selective for K$^+$ than for Na$^+$, and the Na$^+$ can be displaced by K$^+$.

The complexity of the Na$^+$ and the K$^+$ activation and interaction is becoming apparent. Na$^+$, in the presence of low K$^+$ concentrations, stimulates phosphatase activity; but, in the presence of equi-effective concentrations of activating cations, such as K$^+$, NH$_4^+$, and Th$^+$, Na$^+$ does not stimulate. This observation would also support the theory of co-existing Na$^+$ and K$^+$ sites.

In the phosphatase reaction in red blood cell ghosts, Na$^+$ without ATP is a competitive inhibitor for K$^+$; but with ATP, Na$^+$ strongly inhibits the K$^+$ activation (94). When the ghosts are placed in a Na$^+$-containing medium, the phosphatase does not hydrolyze *p*-NPP even in the presence of K$^+$, and the simultaneous presence of ATP and *p*-NPP does not initiate the efflux of Na$^+$ from the ghosts (11). This interaction could be explained by considering two reaction pathways when a weakly activating substrate, such as *p*-NPP, is present instead of ATP or CTP:

1. the Na$^+$ - K$^+$ Pathway, corresponding to the ATPase reaction, would predominate when both cations were present in the medium, especially if some ATP were also introduced.
2. the K$^+$-Pathway, corresponding to the phosphatase reaction, would predominate in the absence of Na$^+$ (117). This is

compatible with the theory of direct access of artificial substrates to the K$^+$-activated hydrolytic site without prior phosphorylation of the enzyme in a Na$^+$-dependent step (see section 3).

In terms of Robinson's kinetic analysis, two models can account for the ATPase and phosphatase kinetics with respect to Na$^+$ and K$^+$ activation:

1. an allosteric model with multiple interacting sites, and;
2. a multiple site - multiple affinity model with competitive interactions.

Skou (135) has also examined the complex interdependence of these ions. He noted that when the K$^+$:Na$^+$ ratio was low, ATP enhanced phosphatase activity, but at higher K$^+$:Na$^+$ ratios, ATP was not necessary to enhance activity (Fig. 5, Page 23). Since Na$^+$ alone had no effect, this phenomenon was attributed to the combined effect of both cations, again attesting to the complex nature of the reaction.

(c) Magnesium ions

Another ion which plays an important role in the phosphatase reaction (as well as in the kinase reaction) is Mg^{++}. While it must be present internally, its presence externally does not alter the reaction (105). In the (Na$^+$ + K$^+$)-ATPase system, Mg^{++} has a rôle in forming the Mg^{++} - ATP complex which is thought to be the effective substrate for the Na$^+$-dependent phosphorylation reaction. This complex does not form if EDTA is present in the medium or if the concentration of Mg^{++} is very low (5, 23, 148). Although Mg^{++} is not necessary for the K$^+$-activated hydrolysis of the phosphorylated enzyme intermediate, it is necessary for the K$^+$-activated hydrolysis of artificial substrates by the membrane phosphatase. Apparently Mg^{++} combines with a site which is independent of the substrate or K$^+$-reacting sites, and does not interact with them. The exact physiological

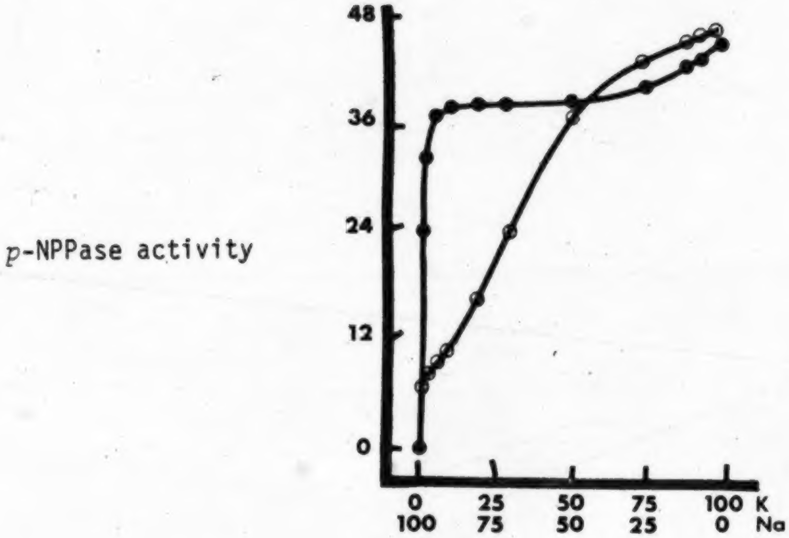


Fig. 5. The effect of Na^+ and K^+ on *p*-NPPase activity with (●) and without (o) 0.1 mM ATP. (The sum of the concentrations of K^+ and Na^+ was kept constant at 100 mM.)

meaning of the internal location of the Mg^{++} site of the phosphatase is obscure (105). However, the formation of an Mg^{++} salt of the artificial substrate and the internal location of the Mg^{++} site seem to strengthen the argument for the internal location of the phosphatase reaction site. Optimum K^+-phosphatase activity was obtained in turtle bladder cells when the Mg^{++} : *p*-NPP ratio was 1:0 (129), and in red blood cells when the Mg^{++} : *p*-NPP ratio was 3:50 (52).

The requirement for Mg^{++} in the *p*-NPPase reaction appeared to be absolute in rabbit kidney (148). The concentration of Mg^{++} necessary to achieve maximum activation increased as the K^+ concentration increased. When the concentration of Mg^{++} exceeded that of K^+, phosphatase activity was inhibited. This implied that the activating effects of K^+ and Mg^{++} follow cooperative kinetics, and that K^+ activates the reaction when it is present in a concentration sufficient to overcome the effects of Mg^{++} inhibition. A slight activation by K^+ at low Mg^{++} concentrations exhibited non-cooperative kinetics. In the absence of inhibitory Mg^{++} binding, when the K^+ concentration exceeded the Mg^{++} concentration, the enzyme was said to be in a "Mg-regulatory conformation" which was less sensitive to inhibition by ouabain. In the presence of inhibitory Mg^{++} binding, the enzyme was in a "K^+-regulatory conformation" which was ten times more sensitive to inhibition by ouabain. At pH 7.4, the interconversion of the two conformations occurred when the concentrations of Mg^{++} and K^+ were equal. However, the critical ratio of Mg^{++} : K^+ concentration varied with pH.

The relationship between Mg^{++} and ATP was examined by Skou (135) in 1974. In the presence of optimal Na^+ and K^+ concentrations, a high Mg^{++} : ATP ratio inhibited (Na^+ + K^+)-ATPase activity and also the *p*-NPPase activity. This was consistent with results of Tashimo *et al.* (148). Mg^{++} - ATP would not seem to be the common factor for the inhibition of (Na^+ + K^+)-ATPase because, at a low Mg^{++} : ATP ratio, the amount of Mg^{++} - ATP present was less, but (Na^+ + K^+)-ATPase activity was enhanced, while the *p*-NPPase activity was lowered. These observations implied that the enzyme possessed a rather high affinity for activation by free ATP. Alternatively, ATP could form a complex with Mg^{++} as well as compete with *p*-NPP. Skou still debated whether there was a separate activator site for free ATP besides a substrate site, or whether free ATP was the necessary substrate for ATPase activity (135).

(d) Calcium and other ions

Another divalent cation which exerts special effects on (Na^+ + K^+)-ATPase activity is Ca^{++}. In the presence of ATP and K^+, it was found to activate the enzyme of the red blood cell (99). This activation was specific for ATP since the other nucleotides maintained low activity. EGTA abolished this increase in ATP turnover rate, but it either did not alter or it enhanced other Ca^{++}-dependent enzyme effects. Therefore, ATP could combine with the enzyme in the same manner with or without Ca^{++}. This implied that ATP binding was at a different site, and Ca^{++} activation was due to a change induced by the nucleotide in the reactivity of the enzyme towards the cation. Thus, ATP could competitively inhibit Ca^{++} activation.

Since the activation of both ATPase and phosphatase occurred when Ca^{++} was present in the interior of red cell ghosts, it was then postulated that both activations were related phenomena, perhaps by sharing of the same ATP and Ca^{++} sites. This hypothesis supported the concept that both activities were due to the same enzyme complex (107).

Ca^{++}, Sr^{++}, and Ba^{++} could induce a large increase in the K^+-dependent phosphatase activity, associated with a reduction in the ouabain-sensitive phosphatase activity (99) but only in the presence of ATP. Ca^{++} decreased, but did not abolish, the ATP-dependent rise in the apparent affinity of the phosphatase for K^+. A Ca^{++} concentration in the medium between 0.5-0.7 mM could enhance K^+-phosphatase activity, especially in the presence of K^+ and ATP. At higher Ca^{++} concentrations, the phosphatase activity slowly decreased under any conditions.

It is interesting that while Th^{++} has ten times the affinity for the K^+ site than does K^+ itself, it can activate *p*-NPPase activity to only 85% of that level which an equimolar concentration of K^+ could achieve.

(e) Ion transport

The activation of (Na^+ + K^+)-ATPase by Na^+ and K^+ ions is intimately related to their transport across a biological membrane. The formation of the high-energy phosphorylated intermediate by the kinase activity of ATPase induces a conformational change in the complex which is associated with the active transport of cations. The best tissues to use for ion transport studies are the intact red cell and a ghost preparation. In these tissues the internal and external compartments are naturally present, all the ATPase is properly oriented within the membrane, and cation movements between the compartments can be easily observed. Whether or not an artificial phosphatase substrate can drive cations through an active transport system is still a controversial subject. Because *p*-NPP and other acetyl phosphates phosphorylate at the $E_2 \sim P$ stage, they would not be expected to drive a Na^+ - K^+ or a Na^+ - Na^+ exchange through the "Pump". If the concentrations of *p*-NPP were increased to saturate its binding sites, the system would become progressively trapped in the $E_2 \sim P$ form and an ATP-dependent exchange would be progressively inhibited (52).

When the Na^+ concentration was high and the K^+ concentration was low, Skou (135) found that the phosphatase reaction did not give a cycling of the carrier sites between the internal and external environment, and that cations were not transported. The combined effect of the ions suggested that Na^+ and K^+ sites existed simultaneously. An increase in phosphatase activity would then be due to the replacement of Na^+ on its own site by K^+. The apparent affinity for K^+ at the Na^+ site in the presence and absence of ATP was 2.3 and 2.5 times higher respectively than the affinity for Na^+. Since ATP did not greatly alter the affinity for K^+, it could have been enhancing the catalytic activity of a low activity form of the enzyme more than was *p*-NPP. K^+ seemed to be necessary internally on the Na^+ site and externally on the K^+ site for phosphatase activity. When the $K^+ : Na^+$ ratio was increased, the phosphatase activity was enhanced probably by the conversion from a low to a high activity form of the enzyme.

On the other hand, Askari and Rao (14) found that *p*-NPP could stimulate Na^+ efflux from red cells, but only if it were present in the external medium. They found that Na^+ efflux was a product of the apparent internal Na^+ concentration and the outward rate constant and could be inhibited by only a small amount of Na^+ in the medium. The magnitude of the efflux also depended upon the external *p*-NPP concentration, and, hence, was sensitive to *p*-NP production. The inhibition by external Na^+ at all K^+ concentrations could be overcome by the addition of ATP to the medium. However, in an Na^+-free medium, ATP had no significant effect of Na^+ efflux. Small amounts of orthophosphates could counteract the stimulation of Na^+-efflux by *p*-NPP.

In summary, these experiments showed that:

1. both Na^+ and K^+ translocation were associated with a K^+-dependent phosphatase segment of the $(Na^+ + K^+)$-activated-ATPase complex;
2. the primary role of the ATP-protein kinase "segment" or the "transphosphorylation segment" (14) was to produce a substrate

for the translocator phosphatase;

3. the substrate (E $\sim$ P) production step was controlled by the internal Na^+ concentration.

5. Inhibitors

Inhibitors are used as aids in investigating the biochemical and physiological mechanisms of action of enzyme systems. One group of inhibitors which has already been discussed above are the ions which influence the (Na^+ + K^+)-ATPase and K^+-phosphatase enzyme functions. Whether these ions act as either stimulators or inhibitors depends on their concentrations and other assay conditions. Another more important group of inhibitors is the cardiac glycosides. Of these, ouabain is the one most frequently used in laboratory studies because of its greater water solubility compared to other members of the family, such as digoxin, digitoxin, etc.

(a) Cardiac glycosides

The cardiac glycosides have enjoyed wide clinical application for several centuries. Only in the past two decades has the interest in their actual mechanism of alleviating congestive heart failure been investigated. The cardiac glycosides are known to inhibit the hydrolytic and transport functions of (Na^+ + K^+)-ATPase, and a reasonable correlation has been achieved between this inhibition and the positive inotropic effects in the heart but, nonetheless, this postulate is still under scrutiny (73, 120). As early as 1965, Repke (104) had suggested that the arrythmias induced by cardiac glycosides were also due to the inhibition of (Na^+ + K^+)-ATPase. Unfortunately, this theory could not be easily proven because of the highly tenacious binding of the glycosides to the enzyme. The introduction of the semisynthetic glycoside, actodigin (168), facilitated such an investigation because it could dissociate from the enzyme within an experimentally measurable period of time. Infusions of this drug into dogs demonstrated that actodigin-induced arrhythmias paralleled (Na^+ + K^+)-ATPase inhibition. Therefore, the change in the electrical activity in the heart induced by cardiac glycosides can now more confidently be ascribed to the inhibition of (Na^+ + K^+)-ATPase.

The following discussion will now focus primarily on the *in vitro* use of cardiac glycosides to examine the biochemical functioning of the (Na^+ + K^+)-ATPase reaction and of the K^+-phosphatase reaction, and will not deal with its clinical use.

Cardiac glycosides were shown to inhibit the overall (Na^+ + K^+)-ATPase reaction, therefore, their exact locus of inhibition in the reaction sequence was sought. Each step of the sequence could be shown to be inhibited, but the inhibition of the phosphatase reaction specifically could account for the inhibition of the other steps. Inturrisi and Titus (65) suggested that ouabain inhibited the reaction by binding to the phosphorylated enzyme intermediate, thus forming what they called an $E \sim P \sim O$ complex. Ahmed and Judah (1), in 1964, were first to suggest that cardiac glycosides specifically inhibited the phosphatase reaction when they observed that the activity of the phosphatase decreased in parallel with that of the (Na^+ + K^+)-ATPase after treatment with strophanthin G (ouabain). If each step is considered individually, the

the formation of an irreversible E ~ P ~ O complex would:

1. block the K^+-induced loss of phosphate from the enzyme in the presence of Mg^{++} and Na^+;
2. retard the maximal rate of K^+-independent loss of ^{32}P from the enzyme;
3. prolong the time required for the maximal level of ^{32}P labelling of (Na^+ + K^+)-ATPase in the presence of Mg^{++} and Na^+ (the "ouabain shift" of Shamoo (121, 122)).

As a consequence of inhibition at this level, the Na^+-dependent formation of the phosphorylated-intermediate would also be inhibited. Charnock and Potter (29) and Britten (24) presented evidence that the Na^+-dependent phosphorylation reaction is directly inhibited by ouabain (perhaps by the formation of an E ~ O complex). This direct inhibition was shown to be maximal when concentrations of Na^+ and Mg^{++} ions were optimal for maximum phosphorylation (29). The overall result by either means would be the inhibition of Na^+ and K^+ transport.

The above models would imply that the phosphatase enzyme is a part of the same enzyme complex as the (Na^+ + K^+)-ATPase molecule. Brooker and Thomas (25) disagreed with this opinion after obtaining biochemical data using a physiological model. In perfused frog heart ventricles, the inhibition of (Na^+ + K^+)-ATPase during the positive inotropic action increased as the inotropism increased, but the inhibition of the phosphatase did not occur at the same ouabain concentrations used to inhibit the overall (Na^+ + K^+)-ATPase.

A significant concentration effect has been observed by several investigators. The concentration of ouabain required to inhibit the partial phosphatase reaction was greater than the concentration required to inhibit the overall (Na^+ + K^+)-ATPase reaction (96). Fujita *et al.* (48) found that thirty times the concentration was necessary to inhibit the phosphatase from pig cerebrum, and in our own work, using the enzyme from beef brain microsomes, five times the concentration of ouabain was used to inhibit the phosphatase reaction relative to the overall (Na^+ + K^+)-ATPase reaction. It is rather puzzling that a partial reaction should require so much more drug to be inhibited than the overall reaction! The inhibition of the overall reaction might be occuring by the formation of an $E_1 \sim O$ complex as well as by an $E_2 \sim P \sim O$ complex, the latter complex would then reflect the inhibition of the phosphatase reaction, specifically. The affinity of the E_1 form of the enzyme under optimal phosphorylation conditions might be higher than the affinity of the $E_2 \sim P$ form, and thus the E_1 form would require a smaller inhibitory concentration of ouabain.

The competition between ouabain and K^+ for the same active site was mentioned in section 4 (a). Two binding sites have been proposed to exist for K^+, a regulatory site and a catalytic site (4, 58). Recently, two ouabain binding sites were suggested to exist (35, 55, 60) with different affinities at each site for substrates and ligands affecting ouabain binding. Godfraind *et al.* (55) stated that the high-affinity component of binding was observed at lower ouabain concentrations. Hansen (60) and Charnock (35) both stated that the sites exhibited homogeneity with respect to g-strophanthin affinity after the addition of K^+, which would imply that the extent of competition at each site was equal and that the two binding sites were different states of the same population of receptors. Studies in our laboratory have shown that only one of the binding sites was associated with a lipid moiety in the membrane which was sensitive to lipolytic attack, and was the site responsible for the "receptor" properties of the $(Na^+ + K^+)$-ATPase. The other site was relatively resistant to attack by both detergents and lipases.

Ca^{++} is the only other ion besides K^+ which has demonstrated the ability to induce changes in the ouabain inhibition of the $(Na^+ + K^+)$-ATPase enzyme complex. At concentrations from 0.5 to 0.7 mM, Ca^{++} is known to stimulate the phosphatase reaction as well as the overall $(Na^+ + K^+)$-ATPase reaction (97). Higher concentrations of Ca^{++} inhibit the activities. The simultaneous presence of ouabain with a high concentration of Ca^{++} would make the inhibition additive (since the binding sites are different). This would explain the extreme toxicity manifest clinically as arrhythmias, when the two substances are administered together.

While ouabain inhibited the $(Na^+ + K^+)$-ATPase and K^+-phosphatase, it also activated the Mg^{++}-phosphatase, especially in the absence of K^+ (96). The same binding site was proposed to be responsible for all the effects on the three enzyme reactions, as well as the same mechanism of action, i.e., the conformational change induced in the enzyme by the binding of ouabain (96). Godfraind *et al.* (55), however, proposed that the low affinity binding site was responsible for the activation of the "Sodium Pump". Ouabain must have bound to the enzyme to form an $E \sim O$ complex before the activation could occur. The extent of activation by ouabain as determined from its linear rate of binding was a measure of the amount bound. Concentrations of ouabain greater than 1 mM maximally activated the Mg^{++}-dependent activity and inhibited the K^+-stimulated activity. Ouabain activation was observed at suboptimal substrate concentrations (0.2 mM ATP was used in the experiments of Godfraind). At a low ouabain concentration, in the presence of a low ATP concentration, ouabain activation was greatly increased, probably due to the enhanced affinity of the ATPase for ouabain. At ATP concentrations above 0.2 mM, ouabain inhibition was seen instead. ATP was the most effective nucleotide to stimulate an ouabain-binding which activated the phosphatase reaction. Na^+ inhibited the ouabain activation of *p*-NPPase by the nucleotides, but activation was still observed in the presence of ATP even if the ouabain concentration was below that required for maximum activation in the presence of ATP alone in the absence of Na^+. This significant stimulation suggested that phosphorylation was not a prerequisite for ouabain binding (97).

Changes in ouabain binding and inhibitory effects due to manipulations of membrane lipids will be discussed in section 6, pp. 38-57.

Other physiological effects have been indirectly affected by ouabain inhibition. These include chemotaxis in neutrophils (154), catecholamine-induced lipolysis in adipose tissue (166), and inhibition of glycerol release by xanthines (166).

The presence of cardiac glycosides or the absence of K^+ in the medium were implicated in blocking the catecholamine induced lipolysis in adipose tissue (166). The antilipolytic affect of cardiac glycosides was due to the inhibition of noradrenaline uptake mediated by the inhibition of transport ATPase in the adipocyte membrane. The same mechanism under the same conditions was implied for the inhibition of glycerol release induced by theophylline and other xanthines.

(b) Other inhibitors

The following agents were shown to inhibit the phosphatase reaction indirectly by influencing either the steps preceding or following it and not specifically inhibiting the phosphatase step as did the cardiac glycosides. These inhibitors differ also because they are not used clinically as are the cardiac glycosides.

OLIGOMYCIN inhibits (Na^+ + K^+)-ATPase, but not K^+-phosphatase specifically. Similarly, N-ETHYL MALEIMIDE (NEM) inhibits (Na^+ + K^+)-ATPase to a greater extent than K^+-phosphatase.

Oligomycin never inhibits ATPase completely even at high concentrations, but inhibition is enhanced at low temperatures. Na^+, even in low concentrations, is necessary for oligomycin inhibition of (Na^+ + K^+)-ATPase and also of K^+-*p*-NPPase (10, 12, 118, 164). The presence of Na^+ also requires ATP, but low concentrations of ATP can reduce oligomycin inhibition. High Mg^{++} concentrations also reduce oligomycin inhibition.

The mechanism by which ologomycin lowers (Na^+ + K^+)-ATPase activity is thought to be by shifting the equilibrium between the alternate allosteric forms of the enzyme, i.e., blocking the $E_1 \sim P \longrightarrow E_2 \sim P$ conversion. This shift in equilibrium is opposed by Mg^{++} and by high temperatures, both of which promote the forward reaction. Na^+ is necessary for oligomycin binding, for the allosteric changes, and for the operation of (Na^+ + K^+)-pathway of the phosphatase to expose the ologomycin effects. Oligomycin does not block active cation transport completely, although it lowers the number of K^+ ions transported per ATP molecule hydrolyzed (67, 118, 120). The inhibition of the $E_1 \sim P \rightleftharpoons E_2 \sim P$ conversion without the block of active transport implies that enzyme conversion may not be an essential part of the translocation scheme, which is contrary to current accepted theories of transport.

Low concentrations of oligomycin, in the presence of Na^+ and K^+ were found to have an activating effect on the phosphatase reaction. This was attributed to the ability of oligomycin to overcome the effects of Na^+ on the apparent Km of K^+ (10). An optimum concentration of Na^+ was necessary for the activating effects which resembled those produced by ATP except that the maximum activation by oligomycin never approached that by ATP. A high concentration of oligomycin could not inhibit the K^+-phosphatase as did a high concentration of ATP. Similar activating effects were observed with only one other inhibitor, RUTAMYCIN. This behaviour was explained by the Na^+-dependent binding of oligomycin or rutamycin to the same modifying site as ATP, thereby inducing an increase in their activating effects at low K^+-concentrations (10).

HYDROXYLAMINE was also found to inhibit at the $E_1 \sim P \rightleftharpoons E_2 \sim P$ conversion step by irreversibly displacing the phosphate transferred from ATP to the membrane protein in the presence of a small amount of Ca^{++} (50).

DIISOPROPYLPHOSPHOFLUORIDATE (DFP) was found to inhibit the K^+-*p*-NPPase more than the $(Na^+ + K^+)$-ATPase. Reaction with a serine group at the active site required Mg^{++}, was potentiated by K^+, and was prevented by a low concentration of ATP (47).

Robinson (113) also applied PHLORIZIN and PHLORETIN to this enzyme system and found, in both cases, that K^+-phosphatase was stimulated (Km = 0.05 mM and 0.01 mM, respectively) and that $(Na^+ + K^+)$-ATPase was inhibited (Ki = 0.06 mM and 0.01 mM, respectively). Neither drug measureably affected the $(Na^+ + K^+)$-independent ATPase. He suggested that phlorizin acted as a heterotropic allosteric modifier of the Na^+-dependent phosphorylation step by lowering the affinity of this step toward Na^+, and enhancing the apparent affinity of the enzyme towards K^+, thereby stimulating K^+-phosphatase activity. This behaviour implied that the phosphatase reaction represented an aspect of the overall ATPase reaction. Robinson suggested that reagents such as phlorizin which can influence the Na^+ affinity at one site on the Sodium Pump might also influence the Na^+ affinity at another site, on a homologue such as the Sugar Pump (113).

PARA-CHLOROMERCURIBENZOATE (PCMB) inhibited all Mg^{++}-*p*-NPPase activity except alkaline Mg^{++}-insensitive-*p*-NPPase (41). Stefanovic *et al.* (139) found that PCMB inhibited 93% of K^+-*p*-NPPase and only slightly inhibited

Mg^{++}-*p*-NPPase of calf thyroid glands while it exhibited the reverse behaviour towards the enzymes of plasma liver membranes. PCMB blocked dephosphorylation in a manner different from that of the cardiac glycosides, but it preferentially inhibited ($Na^+ + K^+$)-ATPase. Tanaka *et al.* (143) found that mercuric chloride itself inhibited ($Na^+ + K^+$)-ATPase and K^+- and Mg^{++}-phosphatases strongly. Concentrations between 10^{-6}-10^{-4}M lowered the activity to 0.4% of the original level.

Although NEURAMINIDASE removed about 70% of the sialic acid of the rat liver membrane and caused a marked inhibition of Mg^{++}-ATPase and also ($Na^+ + K^+$)-ATPase, it completely abolished the activity of Mg^{++}-*p*-NPPase and K^+-*p*-NPPase. Treatment with a ganglioside preparation restored both ATPase activities to 40% and to 65% of control values, but had no effect on the *p*-NPPase activities (41). POLYSINE affected the free carboxyl groups of membrane-bound sialic acid to cause a strong inhibition of K^+-*p*-NPPase and Mg^{++}-*p*-NPPase activities. These results suggested that a glycoprotein conjugate, especially a carboxyl group of a membrane-bound sialic acid, was acting as a K^+ receptor, or as a carrier in the transport of K^+ across the membrane.

FLUORIDE ION enhanced the permeability and lowered the active ion transport of the cell membrane by inhibiting ($Na^+ + K^+$)-ATPase more than K^+-phosphatase (164). Mg^{++} was essential and K^+ also stimulated the inactivation of both enzyme reactions by F^- ion, possibly by causing a structural change in the enzymes or in the membrane in which they were located (47, 164). Such a structural change might be prevented by ATP and by Na^+ which antagonized F^- inhibition (164). Opit *et al.* (93) suggested that inhibition by F^- ions and by increasing Mg^{++} concentrations was additive. Formby (47) also suggested that the site of inhibition was the metal binding site of the enzyme and found that noradrenaline abolished the F^- inhibition of K^+-*p*-NPPase.

DIMETHYLSULFOXIDE (DMSO) caused a reversible, temperature dependent inhibition of $(Na^+ + K^+)$-ATPase activity and a stimulation of the associated K^+-dependent phosphatase activity. Robinson (119) used this agent as an aid in determining if the same site alternately bound Na^+ and K^+, or whether each cation had its own class of site. (see sections 4 (a) and (b), pp. 18 - 23. The pattern of uncompetitive inhibition toward both ATP and Na^+ implied that DMSO influenced the reaction stages following the interaction of the enzyme with Na^+ and ATP. Beyond the $E \sim P$ stage, DMSO could modify the enzyme structure by stablizing an intermediary stage in the sequence of protein conformations. This postulate was supported by the fact that DMSO lowered the affinity for K^+ which was necessary for the subsequent liberation of inorganic phosphate from $E \sim P$. DMSO enhanced the affinity of the phosphatase for artificial substrates by facilitating their entry to the hydrolytic site while hindering the hydrolysis of the normal substrate $E_2 \sim P$. (Perhaps, it interfered with K^+-dependent migration of $E \sim P$ to the hydrolytic site.) DMSO seemed to divert *p*-NPP and artificial substrates from a rate-limiting step of the overall ATPase reaction.

6. The Role of Phospholipids

The concept that the $(Na^+ + K^+)$-ATPase effects are modulated within the membrane matrix by major membrane components such as the various lipids, is generally supported by the evidence of many investigators. Essentially, it was found that the specific activity of the $(Na^+ + K^+)$-ATPase paralleled the phospholipid content of the enzyme preparation, and that activity was consistently restored to lipid-depleted preparations by adding a variety of exogenous phospholipids (63, 121, 156). These observations have generated two specific questions:

1. is there a requirement for a specific kind of phospholipid? and;
2. what is the function of the lipids?

The major methods of investigating lipid-protein interactions required the isolation of a delipidated protein followed by the attempt to reactivate the preparation by various amounts. Lipid interactions with proteins and other lipids can be disrupted by:

1. detergents which alter lipid-lipid interactions in the membrane and necessarily influence the proteins embedded therein;
2. halide-ions, which remove extrinsic proteins thereby disrupting interactions with lipids which they contact, and;
3. lipolytic enzymes, such as the phospholipases which not only disrupt the lipids of the matrix, but appear to be the only agents capable of attacking the boundary lipids surrounding an intrinsic protein. Phospholipase A_1 or A_2 (PPLA) from bee or snake venom hydrolyzes the ester bond between a fatty acid and a glycerol carbon, to produce a free fatty acid and a lysophospholipid. Phospholipase C (PPLC) hydrolyzes the ester bond between the phosphate group and the glycerol carbon, to produce a diglyceride and a phosphorylated product such as choline phosphate or ethanolamine phosphate (136). A diagram of a phospholipid molecule is presented in Fig. 6, Page 40.

Various manipulations of lipid interactions have been performed by several investigators and their findings will now be discussed. The literature was reviewed bearing in mind the questions raised about the function and specificity of the lipids of the K^+-phosphatase reaction, and whether or not this enzyme activity was a part of the $(Na^+ + K^+)$-ATPase complex or a separate entity.

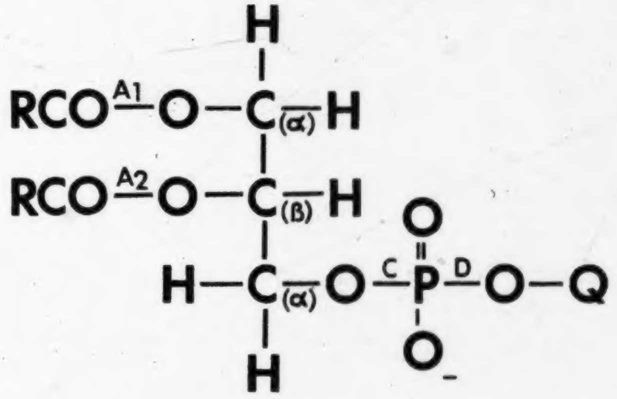


Fig. 6. Diagram of a typical phospholipid molecule. A_1, A_2, C, D, designate the phospholipases which hydrolyze the bonds indicated. R represents fatty acyl moeities; Q represents a polar moiety, e.g., an amino acid.

(a) Lipid-lipid interactions

Lipid-lipid interactions are disrupted by detergents, such as deoxycholate (DOC), sodium dodecyl-sulfate (SDS), and Lubrol. Tanaka and Mitsumata (144) solubilized the lipids of a brain cerebral cortex fraction by DOC treatment and examined the relationship between the solubilized enzyme and the lipids. They found that DOC solubilized all three of the following membrane phosphatases: The acid-phosphatase activity was not lower, but the Mg^{++}-dependent and the Mg^{++}-K^+-dependent phosphatase activities were lower after DOC treatment.

Addition of lecithin (PC) derived from an animal source to the solubilized preparation did not affect the acid- and Mg^{++}-dependent phosphatases, even at high concentrations, but restored the Mg^{++}-K^+-phosphatase to 70% of its original activity. The addition of charged phospholipids stimulated Mg^{++}-K^+-phosphatase two-fold, but unsaturated or neutral lipids and fatty acids inhibited it. Oleic and linoleic acids inhibited the activities almost completely, but monolein reduced them by only 30%, and diolein and triolein inhibited even less. The phospholipids seemed to offer some protection against thermal inactivation, which suggests one further role for the lipids in biological membranes. It was not decided which particular lipid was required for this protection. The requirement in the phosphatase reaction did not seem to be as absolute as that in the overall ($Na^+ + K^+$)-ATPase reaction, or the phospholipids of the *p*-NPPase reaction were more varied than those involved in the ($Na^+ + K^+$)-ATPase activation (143). These studies at least demonstrated that unsaturated and neutral lipids and fatty acids were not required by the systems.

The same species of lipids were not expected to bind to the enzyme system in the same manner as in the intact membrane, after treatment with DOC and lecithin. Since the effects of K^+, Mg^{++} and Ca^{++} ions on the Mg^{++}-K^+-*p*-NPPase were identical in both the intact and solubilized membranes, this inferred that the ion binding sites were not related to the lipid binding ones. This conclusion was similar in the case of the Mg^{++}-(Na^{+}+K^{+})-ATPase where the lipids were also not required for ligand binding. The persistent question of whether the Mg^{++}-K^{+}-*p*-NPPase was an integral part of the Mg^{++}-(Na^{+} + K^{+})-ATPase or whether it was a separate entity was not resolved by these investigations. The results did suggest, however, that the K^{+}-phosphatase could be part of the (Na^{+} + K^{+})-ATPase system with a different, but intimately related lipid effect.

The work of Wheeler *et al.* (155) on (Na^{+} + K^{+})-ATPase activity after extraction of a rabbit kidney preparation with DOC or Lubrol indicated that phospholipid and cholesterol content, and overall (Na^{+} + K^{+})-ATPase activity decreased in parallel, but the activity was completely lost before all the lipid had been removed. The loss of activity could not be attributed to inhibition by residual detergent. There was no evidence for the selective removal of any one type of lipid. Therefore, either the loss of overall (Na^{+} + K^{+})-ATPase activity was not associated with the removal of a particular lipid, or such specific removal was obscured by other contaminating lipids present. Partial purification to give a 50-fold increase in specific activity was not accompanied by selective enhancement of any particular class of phospholipids.

Complete restoration of phosphatase activity was achieved with those preparations in which the residual enzyme activity after detergent extraction was 60-90% of control. There was no correlation between restoration of (Na^{+} + K^{+})-ATPase and K^{+}-*p*-NPPase activities (156). Restoration of the former activity with exogenous phospholipids was consistent, whereas that of the latter was not. Phosphatidylserine and phosphatidylcholine could reactivate the phosphatase, but phosphatidylethanolamine slightly inhibited the activity, while phosphatidylinositol, phosphatidylglycerol, and diphosphatidylglycerol greatly inhibited it. Added phospholipids which reactivated the phosphatase did so only over a limited concentration range; higher concentrations failed to activate and actually inhibited the reaction. Therefore, from this work, there is no definite answer suggested to the question of the function of the phospholipids of the phosphatase reaction. Since their requirements in the phosphatase reaction are so much less stringent than in the overall reaction, perhaps they are not required at all for the same conformational purposes. Such an hypothesis would be logical, since the change in conformation would have already occured in the reaction step previous to the phosphatase step. Then, the lipids of the phosphatase reaction would be required for some other purpose than to aid the conformational change in the enzyme.

Wheeler (157) used the phospholipid requirement to investigate the reaction sequence of ($Na^+ + K^+$)-ATPase and to determine where in the sequence the phospholipids may be specifically necessary. A Lubrol extracted preparation could undergo Na^+-dependent phosphorylation by ATP, even though the ($Na^+ + K^+$)-ATPase activity was almost abolished. However, after repeated Lubrol treatments, the enzyme could not be phosphorylated. Therefore, the residual lipid after a single Lubrol extraction was at least necessary for Na^+-dependent phosphorylation. The residual phosphatase activity after a single Lubrol treatment was still 50% of control, and was shown not to be rigidly dependent on lipids. The reaction subsequent to phosphorylation was shown to need exogenous phospholipids. Therefore, the location of the lipid requirement was quite narrowly restricted to the reaction between Na^+-dependent phosphorylation and K^+-dependent dephosphorylation, i.e., the conformational change from the $E_1 \sim P$ to the $E_2 \sim P$ form. Repeated extractions caused either complete or partial denaturation of the enzyme protein itself, due to the removal of the lipids, or, due to the lack of extrinsic proteins which altered the lipid configurations and prevented the conformational change of the enzyme. Wheeler's summary of the phospholipid requirement of the reaction sequence is presented in Table 1 (157). He suggests that some intramolecular transformation or intermolecular coupling process is absolutely dependent on easily removable phospholipids, whereas the initial and final stages of the reaction do not require phospholipid or depend on a limited or a relatively tightly bound fraction. These questions could be answered by reacting exogenous phospholipids with an enzyme preparation which had been completely (or almost completely) depleted of kinase and phosphatase activity by repeated lipid extractions. However, after such drastic treatments, activity has not been successfully restored by exogenous phospholipids. Wheeler's study further confirmed that the phospholipids are required for the maintenance of protein configuration.

The different properties of the ($Na^+ + K^+$)-ATPase and K^+-phosphatase demonstrated after detergent treatment, and by subsequent reactivation, would suggest that either these activities arise from two different enzymes or that the K^+-phosphatase is something other than a partial reaction of the ($Na^+ + K^+$)-ATPase. However, the scheme suggested by Wheeler (Table 1) proposes a mechanism by which the lipid effects can be explained in terms of the phosphatase being a partial reaction of the ($Na^+ + K^+$)-ATPase.

(a) Lipid-extrinsic protein interactions

Membrane extrinsic proteins can be removed by treatment with detergents or with halide ions, e.g., I^-, Br^-, F^-. Fujita *et al.* (48) have treated with NaI alone and Wheeler's preparations have been pretreated with NaI before detergent treatment. Neither of these investigators compared the activities of their NaI treated preparations with an untreated preparation. In the work of Wheeler *et al.* (155, 156, 157) the added NaI treatment does

TABLE 1

Phospholipid requirements of the $(Na^+ + K^+)$-ATPase reaction sequence*

	Reaction	Phospholipid requirement
1.	ATP binding and $(Mg^{++} + Na^+)$-dependent phosphorylation	Either not dependent on lipid or dependent on tightly bound lipid.
2.	Transformation of phosphorylated forms or coupling to K^+-dependent phosphatase	Phospholipid is essential.
3.	K^+-dependent phosphatase	Either not dependent on lipid or dependent on tightly bound lipid.

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*Taken from Wheeler (1975). Full details are given in reference no. 157.

not change the quality of the interactions between the lipids and (Na^+ + K^+)-ATPase nor the conclusions about the phospholipid requirements.

(c) Lipid-intrincis protein interactions

Interactions between intrinsic proteins and boundary lipids can be disrupted by the lipolytic enzymes, phospholipase A (PPLA) and phospholipase C (PPLC). These lipases will disrupt lipid-lipid, and lipid-extrinsic protein interactions, as well.

Taniguchi *et al.* (147) treated ox brain microsomes with PPLA alone and found that 30% of the membrane phospholipids remained intact and that (Na^+ + K^+)-ATPase activity was lowered to 5-25% of control. Colard-Torquebioeu (34) determined that PPLA acted primarily on the interior of platelet cell membranes.

Goldman and Albers (57) treated Electrophorus electric organ with PPLA to investigate the role of phospholipids in the (Na+ + K^+)-ATPase reaction and in the K^+-*p*-NPPase reaction. They found that PPLA treatment resulted in the parallel decrease in both enzyme activities and in the ability to form the phosphorylated intermediate $E \sim P$. Under the conditions of their experiments, inactivation was complete within one hour. The reduction in activities was also paralleled by the removal of phosphatidylserine and phosphatidylethanolamine from their preparations and could not be attributed to the release of fatty acids or to lysophosphatide inhibition.

When Roelofsen and van Deenen (121) treated red cell ghosts with phospholipase A_2, all (Na^+ + K^+)-ATPase activity was completely abolished, and all membrane phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS)were hydrolyzed. Mg^{++}-ATPase activity declined to 0-15% of control. Therefore, lipids closely associated with (Na^+ + K^+)-ATPase and K^+-phosphatase and which are substrates for PPLA are also responsible for maintaining enzyme activity.

When Taniguchi and Tonomura (147) treated ox brain microsomes with PPLC, the amount of phospholipids decreased to 40% of control, and the $(\mathrm{Na}^+ + \mathrm{K}^+)$-ATPase activity also decreased to 40% of control while the K^+-*p*-NPPase activity declined to 70% of control. The major loss of enzyme activity took place during the first five minutes of treatment. PPLC was postulated to act primarily upon the external cell surface (36). When Roelofsen and van Deenen (121) treated red cell ghosts with PPLC, $(\mathrm{Na}^+ + \mathrm{K}^+)$-ATPase activity was completely abolished and Mg^{++}-ATPase activity was reduced to 5-30% of control. Phosphatidylcholine and phosphatidylethanoamine were completely hydrolyzed while only 0-5% of the phosphatidylserine was hydrolyzed.

Phospholipase C from different sources possesses different substrate specificity, the most varied being from *Cl. perfringes* and *B. cereus* (81, 141, 142). The PPLC from *Staph. aureus* did not require Ca^{++} for its action, was inhibited by cations, and was active upon the phosphatidylinositol (PI) of sonicated lipid extracts. PPLC from lymphocytes hydrolyzed *pure* phosphatidylinositol, but not that found in red cell ghosts or in liver microsomes (81). Thus, van Deenen (121) believes that much of the discrepancy in the results in the field of lipid investigation is due to the different sources and, hence, purities and substrate specificities of PPLC which have been employed by various investigators. Therefore, he, quite logically, insists on purifying all the lipases used in his laboratory before applying them to membrane preparations.

A somewhat different result was obtained when Taniguchi and Tonomura (147) treated ox brain preparations with both phospholipases A and C simultaneously. The $(\mathrm{Na}^+ + \mathrm{K}^+)$-ATPase activity was lowered to 25% of control while the *p*-NPPase activity was only slightly lowered to 70% of control. Therefore, the lipids associated with the phosphatase reaction were also responsible for maintaining its activity.

Roelofsen and van Deenen (121) treated red cell ghost preparations also with sphingomyelinase and phospholipase D (PPLD). Sphingomyelinase converts sphingomyelins to ceramides which remain bound to the membrane. This treatment resulted in an increase in ($Na^+ + K^+$)-ATPase activity to 120-140% of control and no change in the Mg^{++}-ATPase activity. PPLD can attack phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, converting them to phosphatidic acid and thereby increasing the net negative charge in the membrane. There was some increase in the ($Na^+ + K^+$)-ATPase activity and a drastic reduction in Mg^{++}-ATPase activity which suggested that Mg^{++}-ATPase activity was particularly sensitive to the membrane charge. When applying phosphatidylserine decarboxylase to red cell ghosts (to convert phosphatidylserine to phosphatidylethanolamine) the ($Na^+ + K^+$)-ATPase activity was not significantly altered while 12-15% of the phosphatidylserine of the membrane was still present. When this last portion was removed by a second treatment with PPLC which had been preceeded by an extraction with dry ether, ($Na^+ + K^+$)-ATPase activity was abolished while Mg^{++}-ATPase activity did not change significantly from the control values (Table 2), Page 49.

Therefore, while the results from delipidating treatments have shown that lipids are necessary to maintain enzyme activity, they also suggested that certain specific lipids might be responsible for that activity. Reactivation experiments where lipid content is restored to extracted preparations are more capable of demonstrating which specific lipids are involved and also their role in the various steps of the reaction sequence.

(d) Reactivation of lipid-depleted enzyme preparations

The ability to reactivate the enzyme after PPLA treatment depended upon the amount of residual activity, which itself depended upon the duration of treatment. If greater than 40% of the activity was lost, then reactivation was not possible (147). The ($Na^+ + K^+$)-ATPase activity of

TABLE 2

Effects of lipase treatment on membrane constituents

Lipase treatment	$(Na^+ + K^+)$-ATPase Activity % control	K^+-*p*-NPPase Activity % control	Residual Membrane Lipids	Ref.*
PPLC	40	70	40% total	(147)
PPLC	----	5-30	0% PC 0% PE 0-5% PS	(121)
PPLA and PPLC	25	70	----	(147)
Sphingomyelinase	120-140	----	----	(147)
Phosphatidylserine Decarboxylase	100	----	12-15% PS	(121)

*Full details of these references are given in the Bibliography section of this thesis.

delipidated preparations could be restored to 50% of control by reconstituting with phosphatidylinositol, and to 100% by reconstituting with phosphatidylinositol plus phosphatidylserine, or by phosphatidylinositol plus phosphatidylserine plus phosphatidylethanolamine (147).

When Taniguchi and Tonomura (147) reintroduced phosphatidylserine to an ox brain preparation delipidated by PPLC, the ($Na^+ + K^+$)-ATPase activity was restored to 85% of control. When Roelofsen and van Deenen (121) performed the same procedure in red cell ghosts, the ($Na^+ + K^+$)-ATPase activity was restored to 50% of control. Phosphatidic acid was also effective, but not as efficient, in restoring ($Na^+ + K^+$)-ATPase activity to red cell ghosts. Larger amounts of it created a net negative charge which abolished Mg^{++}-ATPase activity. Addition of an aqueous dispersion of erythrocyte total lipid mixtures, phosphatidylcholine, or phosphatidylethanolamine to PPLC-treated ghosts did not restore ($Na^+ + K^+$)-ATPase activity completely, and slightly enhanced Mg^{++}-ATPase activity which retained a larger residual activity in the first place. However, Taniguchi and Tonomura (147) found that phosphatidylethanolamine could completely restore ($Na^+ + K^+$)-ATPase activity to delipidated ox brain preparations.

Attempts by Roelofsen and van Deenen (121) to enhance ($Na^+ + K^+$)-ATPase activity of PPLC-treated red cell ghosts beyond 50% of control by increasing the lipid:protein ratio were not successful. DOC treatment after PPLC treatment restored the activity to only 50% of control, as well. Sphingomyelin and cholesterol were incapable of maintaining activity on their own, and the absence of sphingomyelin actually enhanced activity. Removal of diglycerides and cholesterol by dry ether extraction after PPLC treatment left sphingomyelin as the only lipid in the membrane. Treatment of this preparation with phosphatidylserine fully restored ($Na^+ + K^+$)-ATPase activity. Total lipid extracts also could significantly reactivate this preparation, but phosphatidylethanolamine could not reactivate it beyond 10% of control.

When Taniguchi and Tonomura (147) added phosphatidylethanolamine to ox brain enzyme preparations delipidated by PPLC plus PPLA, the $(Na^+ + K^+)$-ATPase activity was reactivated to 65% of control. Lysophosphatidylcholine slightly reactivated the preparation, but large amounts suppressed it further. Although phosphatidylinositol could reactivate $(Na^+ + K^+)$-ATPase after PPLA treatment alone, it was unable to do so after treatment with both agents. The slight reactivation by phosphatidylserine could not be enhanced further by adding phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, lysophosphatidylcholine, or sphingomyelin. Complete reactivation of the phosphatase activity was achieved by adding phosphatidylserine. As with the detergent treated preparations, the extent of reactivation after lypolytic enzyme treatment depended on the amount of residual lipids. The success of the different lipids used for reactivation led to speculation of their specific function in the overall $(Na^+ + K^+)$-ATPase and partial reactions.

Taniguchi and Tonomura (147) proposed the following mechanism for lipid modulation: The acidic phosphatidylserine influenced the $(Na^+ + K^+)$-ATPase reaction at the Mg^{++}-binding site, forming a stable and functional configuration for the Na^+-dependent hydrolysis. The stability of the Mg^{++}-PS-complex on the enzyme was then modified by the interaction of cation groups e.g., those of phosphatidylethanolamine, which controlled the affinity of the enzyme for Mg^{++}.

The removal of 80-85% of the neutral phospholipids did not impair the ability of the enzyme to maintain the conformational state necessary for $E \sim P$ formation and for ouabain binding. Therefore, PPLC appeared not to affect the lipids involved in the Na^+-dependent nucleotide exchange reaction, or ATP hydrolysis. The removal of neutral phospholipids enhanced the affinity of the $E_1 \sim P$ form for Mg^{++} (perhaps by increasing the binding strength of the enzyme for Mg^{++}). Phosphatidylcholine could antagonize the above effect by reversing the effects of a high Mg^{++} concentration, by causing an increase in the nucleotide exchange rate and a decrease in the $E_1 \sim P$ to $E_2 \sim P$ conversion.

The next conversion step, involving the conformational change from E_2 to E_1, depended on the dissociation of Mg^{++} from the relatively stable $E_2 \sim Mg$ complex. Therefore, it was found to be more sensitive to Mg^{++}-inhibition in a PPLC treated preparation and was retarded upon the loss of neutral phospholipids also. The decrease in the phosphatase activity which immediately preceeded this conformational change was then associated with the decreased rate of relaxation of E_2 to E_1. Thus, it would appear that neutral phospholipids might hinder Mg^{++} binding, and their removal by PPLC treatment might produce more stable enzyme configurations in the sequence.

Reconstitution of a highly purified ($Na^+ + K^+$)-ATPase from the rectal gland of *S. acanthias* in phospholipid vesicles of varying composition has been carried out in Hokin's laboratory (61, 63). Their philosophy is that lipid specificity in this enzyme system can be studied most easily by reintroducing individual lipids to a completely pure enzyme. The (Na^++K^+)-ATPase prepared in their laboratory is believed to be the purest of any yet attained. The main concern has not been with the activity of the enzyme, but with its ability to transport ions, i.e., they have been separating the reaction mechanism from the transport mechanism. They have demonstrated transport function in crude egg lecithin vesicles and simple phospholipid vesicles which are composed of one or two types of phospholipids with a partially purified enzyme in which endogenous phospholipids were still present. These studies have suggested that phosphatidylcholine was the most important phospholipid necessary for transport. The most recent work (62) has demonstrated coupled

Na^+ and K^+ transport in a pure phosphatidylcholine vesicle containing highly purified (Na^+ + K^+)-ATPase from shark rectal gland embedded in the bilayers. This is in conflict with some of the results cited above, but Hokin suggests that the specificity of phosphatidylserine may not have been for the (Na^+ + K^+)-ATPase activity, but for some other process such as combination of delipidated protein and phospholipid in aqueous solution. He also sites de Pont (38) who likewise gave evidence against phosphatidylserine being essential by converting 99% of it to phosphatidylethanoamine by enzymatic decarboxylation without affecting enzyme reactivity.

Yorio and Bentley (162) also proposed a special role for phosphatidylcholine in ion transport as the site for promoting Na^+-influx without a change in the Na^+-efflux. The cleavage of phosphatidylcholine molecules by PPLC could create free polar groups, thus enhancing the access of Na^+ to the Pump mechanism, and thereby enhancing its influx and creating a "one-way" channel in the membrane.

Goldman and Albers (57) prepared a summary of the phospholipid requirements of the steps of the (Na^+ + K^+)-ATPase reaction sequence which is compatible with the results of other investigators (Table 3), P. 54.

Roelofsen's and van Deenen's (121) finding that a residual 13% of phosphatidylserine remained after lipolytic treatment deserves further comment here. Since such a small fraction was involved in maintaining enzyme activity, it was speculated that perhaps there were two loci of phosphatidylserine localization, and that the orientation of this latter 12-15% is of great importance. Essentially, what was suggested was that an asymmetric distribution of phosphatidylserine was responsible for the different effects. It has been noted that phosphatidylcholine is located mostly on the outside while phosphatidylethanolamine and phosphatidylserine are located on the inside of the cell membrane (121). Therefore, the fact that phosphatidylserine is essential for enzyme activity

TABLE 3.

Phospholipid requirement of the $(Na^+ + K^+)$-ATPase reaction sequence*

	Partial reaction	Phospholipid requirement
1.	$E_1 \text{---} ATP \xrightleftharpoons{Na^+,\ Mg^{++}} E_1 \sim P + ADP$	PS
2.	$E_1 \sim P \xrightleftharpoons{Mg^{++}} E_2 \sim P$	PE (?PC)
3.	$E_2 \sim P + H_2O \xrightleftharpoons{K^+} E_2 + Pi$	PS
4.	$E_2 \xrightarrow{-Mg^{++}} E$	PC?

---

*Taken from Goldman and Albers (1973). Full details are given in reference no. 57.

would suggest that the active site was located internally.

From the experiments described above and from the work of other investigators, many workers have concluded that the role of phospholipids in the ($Na^+ + K^+$)-ATPase reaction is:

1. to impart a negative charge and hydrophobicity to the vicinity of the active site;
2. to maintain a conformation for the Na^+-dependent phosphorylation;
3. to assist in enzyme conversion reactions;
4. to assist in the binding of ions when necessary, and;
5. to impart some thermal stability to the system.

(e) Other methods of lipid modulation

Other methods of manipulating membrane lipid composition have also been attempted, specifically:

1. treatment of leukocytes with the toxin leucocidin (160);
2. altering the lipid composition of the diet of animals (56), and;
3. hibernation, where the lipids are naturally modified to suit an animal's needs (131).

These three methods will be briefly described now.

Leucocidin, a toxin derived from Staphylococci altered the permeability of leukocyte membranes to cations in such a way that it resembled effects in excitable tissue during membrane depolarization (160). It stimulated *p*-NPPase specifically, suggesting that the permeability changes in the cell resulted from structural changes in the Sodium Pump. It was postulated that the ion pump was activated by dissociating the esterified fatty acids from the surface of the *p*-NPPase in the membrane. The properties displayed by the leukocyte phosphatase suggested that it was distinct from ATPase (not as the controversial case with brain and electric organ enzymes, as discussed throughout the introduction). Diisopropylphosphofluoridate and other organic compounds were found to act as non-ionic detergents to enhance the cytotoxic action of leudocidin by preventing the cell from reversing the effects of suboptimal concentrations of leucocidin. These drugs also inhibited chemotaxis and phagocytosis under the same conditions as they enhanced leucocidin activity, which suggested that these physiological phenomena were also influenced by the reversible separation of esterfied fatty acids from *p*-NPPase.

Goldenberg *et al.* (56) studied the effects of altering the lipids modulating the ($Na^+ + K^+$)-ATPase and K^+-phosphatase of rat erythrocytes by administering a fat deficient diet to the rats. Lipids were found to be allosteric modifiers of both enzyme reactions, but their presence or absence did not modify the non-allosteric nature of the enzyme towards the substrate. The equilibrium between the R and T forms of the ($Na^+ + K^+$)-ATPase was altered from control in fat deficient rats (cf. Fig. 4, P. 20). The lipids were demonstrated to be essential in modulating the specific activities of both enzyme reactions. The temperature effects of the enzymes in both fat deficient and sufficient diets were similar between 7-45°C. Phase transitions in erythrocyte membranes from rats fed a fat deficient or a control diet occurred at both 37° and at 30°. However, erythrocytes from fat deficient animals demonstrated a wider range in temperature before the next state became apparent. The activation energies of the ($Na^+ + K^+$)-ATPase activities and of the K^+-phosphatase activities were not significantly different in the erythrocyte preparations from fat-free and from control rats.

Seasonal alteration of membrane lipid composition occurs naturally in animal species which hibernate. Goldman and Albers (58) found that the temperature coefficient of the ($Na^+ + K^+$)-ATPase was lower in the hibernating hamster, but that of the partial reactions, including dephosphorylation, did not differ. They found the most temperature sensitive partial reactions to be the $E_1 \sim P \longrightarrow E_2 \sim P$ conversion and $E_2 \longrightarrow E_1$ conversion, both of which are major conformational events dependent on cation concentrations and lipid states, as already described. Studies by Skoog, Dryden and Charnock on the contractility of the right atrial muscle of the heart of the ground squirrel have suggested that the positive inotropic response to ouabain is greatly diminished during hibernation.

7. Temperature Dependence

The increase in the velocity of a chemical reaction with an increase in temperature was first formally documented by van't Hoff (151) in the nineteenth century. He proposed that the velocity of a variety of organic reactions increased by a factor of two for every 10° rise in temperature. However, when his contemporary, Arrhenius was examining the temperature dependence of several biological processes, he discovered that this relationship did not apply in all cases. Arrhenius devised the following relationship to explain the exponential rise in the velocity of a reaction with an increase in temperature:

$$k = Ae^{-E/RT}$$

where k represents the maximal velocity of the reaction, A represents the Arrhenius constant, E represents the apparent activation energy of the reaction, R represents the gas constant (1.987 cal/mol/°C), and T represents the absolute temperature (°K). The above relationship can be converted to a log form to give the following relationship:

$$\log k = \log A - \frac{E}{2.303\ R} \times \frac{1}{T}$$

Plotting log k vs 1/T has usually resulted in a linear curve with a slope

3

of $E/2.303\ R$ and a y intercept of log A. By calculating values for the slope, a value for the apparent energy of activation, E or Ea, can be determined. $(Ea/2.303\ R = (\Delta \log k/\Delta 1/T))$ This value is not identical to one which would be obtained by directly measuring the heat content of the reaction, but differs by a few hundred calories per mole which is within an acceptable range (103).

Many biological reactions were analyzed in this manner and were thought to consistently yield a linear relationship, especially if the occasional aberrant points were regarded as experimental artifacts. However, there came a time in the early 1960's when the aberrant points could not be ignored and had to be considered as significant (88). This change of opinion resulted in the construction of non-linear, or discontinuous Arrhenius plots. Although, there have been attempts to explain the discontinuities on the basis of thermodynamic properties of an enzyme itself, (20, 41, 69), a simpler, more generally applicable explanation involved the conformational changes of the enzyme within the membrane matrix. This latter model included the participation of the membrane lipids in the change in conformation, or energy level of the enzyme which occurred at the transition temperature of the lipids. Evidence for the existence of two stable conformations of a membrane enzyme has been obtained from differences observed in the sedimentation characteristics, electrophoretic patterns, and thermodynamic properties of the two forms of an enzyme (88) Evidence for a phase transition occurring in lipids of uniform or varied composition has been obtained by examining them through a range of temperatures by electron spin probes, fluorescent probes, or by differential scanning calorimetry. A good correlation has been demonstrated between changes in enzyme activity with temperature and the physical state of the associated membrane lipids (19, 34, 102).

It is therefore reasonable to suppose that two or more separate phases can exist in the membrane and that membrane enzymes can possess independent energies of activation within each phase. This could then explain the observed discontinuities in the Arrhenius plots (161). The temperature range wherein the slope of a non-linear Arrhenius plot changes represents a region where the value for the activation energy is the same for the two different transition states (75, 103). The point of intersection of the two linear curves of a non-linear Arrhenius plot is known as an isokinetic point.

Among biological processes which have been examined by means of an Arrhenius analysis is the functioning of the ($Na^+ + K^+$)-ATPase. Because a good correlation has been demonstrated between the apparent energy of activation of an enzyme reaction and the physical state of its surrounding lipids, the Arrhenius analysis has been the major investigative technique used in the present studies to observe the lipid modulation of the enzyme activity. The apparent energy of activation (Ea) has been considered as the primary parameter to reflect this modulation. The values of Ea_I and Ea_{II} (above and below the inflection point, respectively) and the ratio of $Ea_I:Ea_{II}$ have been used as a basis of comparison of the modulating effects of the membrane lipids after various lipolytic treatments.

Since our laboratory was not the first to employ such techniques, a historical summary of pertinent works will first be presented.

Walker and Wheeler (153) investigated the temperature dependence of the ($Na^+ + K^+$)-ATPase and *p*-NPPase reactions of detergent-treated preparations by means of Arrhenius plots. Little difference was found between ($Na^+ + K^+$)-ATPase activities when assayed by techniques using either ($Na^+ + K^+$)-stimulation or by ouabain inhibition. A non-linear Arrhenius plot of ($Na^+ + K^+$)-ATPase activity was always obtained. The results are consistent with the interpretation that the activation energy of the ($Na^+ + K^+$)-ATPase is influenced by the physical state of the phospholipids associated with the membrane protein. The discontinuity in the Arrhenius plot reflected the gel to liquid-crystalline phase transition of the lipids. After lipid depletion by Lubrol extraction (preceeded by a NaI-treatment), the residual ($Na^+ + K^+$)-ATPase activity of their preparation was too low to permit accurate measurement over the temperature range studied. The temperature dependence of an enzyme reactivated with phosphatidylserine was similar to that which they referred to as the "intact" enzyme and which had, in fact, been treated with NaI.

In contrast, the K^+-dependent and ouabain-sensitive phosphatase activities were not always identical. Under their experimental conditions, ouabain did not completely inhibit the K^+-dependent phosphatase-activity at any temperature. The discrepancy between the activities measured by the different methods became more pronounced as the incubation temperature was lowered. The net result was a linear Arrhenius plot of the phosphatase reaction when determined by K^+-stimulation, but a biphasic curve when determined by ouabain inhibition. However, the biphasic curve was transformed to a linear one when the ouabain concentration was increased from 0.2 mM to 2 mM. After controlled lipid depletion, 50% of the initial phosphatase activity remained, and the K^+-dependent phosphatase activity was only slightly greater than the ouabain-sensitive phosphatase activity at most temperatures. The Arrhenius plots of phosphatase activity measured by both methods gave virtually identical linear results. The effect of temperature on the degree of ouabain inhibition of the phosphatase indicated that the interaction of ouabain with the enzyme was somehow affected by the physical state of the phospholipids present.

It can be concluded that the presence of the phospholipids in the less fluid state below the transition temperature somehow increased the activation energy of the ($Na^+ + K^+$)-ATPase, but not of the phosphatase reaction. This theory suggests that some molecular events such as a conformational change might be involved in the overall ($Na^+ + K^+$)-ATPase reaction, but not in the phosphatase reaction. The conformational change could be confined to only one portion of the overall ($Na^+ + K^+$)-ATPase protein, and that portion would not necessarily contain the K^+-phosphatase active site (assuming that both activities are part of the same enzyme system).

Many enzymes and transport proteins exhibit a similar sudden change in apparent activation energies, without a change in reaction rate, at a particular temperature which may be considered the transition temperature of the membrane. Wynn-Williams (161) investigated these protein-lipid interactions thermodynamically. Applying his membrane model (consisting of a protein phase and a lipid phase) to sarcoplasmic-reticulum Ca^{++}-ATPase in lipid solution, he described the lipid as being of two classes with little contact between each: Class 1 was free in solution (the bulk phase) and Class 2 was associated with the enzyme as an annulus around it (annular or boundary lipids). According to his calculations, the Ca^{++}-ATPase enzyme activity was proportional to the lipid content of the enzyme-lipid solution, or bulk lipids (whose behaviour would then definitely depend on temperature). If the shape of the enzyme molecule changed slightly on formation of the activated state, the enzyme activity would depend on the compressibility of the enzyme-lipid solution. Near their transition temperature, lipids are thought to be highly compressible, so the compressibility of the enzyme-lipid solution might be approximately proportional to its lipid content. The activity of an enzyme in an enzyme-lipid solution might therefore be approximately proportional to the lipid content of the solution, providing a simple explanation for the apparent change in activation energy.

(a) Current studies

The phospholipid requirement of $(Na^+ + K^+)$-ATPase was examined by Charnock *et al.* (32) with Arrhenius plots of untreated and detergent treated beef brain microsomes. Analysis of the untreated control preparations produced a curvilinear plot which could be described as two straight lines intersecting at the putative critical temperature of the mixed membrane lipids. Detergent treatment (DOC, Nonidet- P40) resulted in large increases in enzyme specific activity without changes in apparent activation energies. This observation reinforced a previous conclusion that the thermodynamic parameter of activation energy is independent of the enzyme specific activity and does not reflect the number of active centres which are operational, but rather measures the functional ability of individual sites (32). Because the activation energy does reflect the functional ability of an individual site, it can be influenced by molecular changes within the membrane matrix surrounding a site.

Incubation of the enzyme preparation with phospholipase A (PPLA) produced a marked change in its temperature dependence. Specifically, a linear Arrhenius plot now resulted from an analysis of the activities of the overall $(Na^+ + K^+)$-ATPase reaction between 4 and 37°C. The single activation energy calculated from this linear plot had a value intermediate between the two values above and below the inflection point obtained from a control curve of activity vs. temperature of an untreated enzyme preparation. The linearity could be returned to the non-linear control state by incubating the preparation with phosphatidylserine.

Phospholipase C (PPLC) treatment of an untreated enzyme preparation did not alter the appearance of the Arrhenius plot of $(Na^+ + K^+)$-ATPase. Incubation with phosphatidylserine subsequently produced no change. These results suggested that selective cleavage of the phospholipids involved in the ($Na^+ + K^+$)-ATPase reaction is an important determinant in changing the temperature dependence of the reactions as revealed by the appearance of the Arrhenius plot.

The temperature dependence of the ouabain-insensitive Mg^{++}-ATPase activity was also investigated (30,33).The appearance of the Arrhenius plots and the values of the activation energies were not altered from those of untreated controls after treatment with detergents or either phospholipase A or C.

The phosphatase reaction of the overall ($Na^+ + K^+$)-ATPase reaction was the only partial reaction investigated for changes after lipid modulation by DOC. The activation energies calculated by means of an Arrhenius analysis did not differ appreciably from those of untreated control preparations. The values obtained from curves measuring both K^+-stimulated and ouabain-inhibitable (O.I.) activities are given in Table 4.

The further study of the temperature dependence of the phosphatase reaction after lipolysis of beef brain microsomes with phospholipases A and C is the subject of this thesis. The specific activities and the ouabain inhibition of these preparations at 37°C were also examined. The original questions served to guide the investigation, i.e.:

1. was there a requirement for a specific kind of phospholipid to modulate the phosphatase reaction? and;
2. what was the particular function of the phospholipid(s) in the mechanism of modulation?

The results obtained permit us to speculate as to which lipids are required and to build models of their mechanism of modulation, as others have done. The overall structure and function of the lipid matrix is also under scrutiny and will be discussed later in this thesis.

TABLE 4

Apparent energies of activation of the ($Na^+ + K^+$)-ATPase reaction and of the K^+-*p*-NPPase and O.I.-*p*-NPPase reactions of untreated and DOC-treated beef brain membrane preparations (Charnock, unpublished results).

Enzyme preparation	Ea_I kcal/mole	Ea_{II}	$Ea_I:Ea_{II}$	Tc °C
Untreated:				
($Na^+ + K^+$)-ATPase	14.6	29.8	0.48	21
K^+-*p*-NPPase	8.2	14.5	0.56	23
O.I.-*p*-NPPase	8.2	18.2	0.45	23
DOC-treated				
($Na^+ + K^+$)-ATPase	16.4	45.4	0.36	17
K^+-*p*-NPPase	6.8	19.7	0.34	16
O.I.-*p*-NPPase	8.3	41.8	0.19	14

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METHODS AND MATERIALS

1. Enzyme Assays

(a) Total protein

Total protein content of an enzyme preparation was determined by the method of Lowry *et al.* (82) using bovine serum albumin (BSA) as a reference standard. The assay was based on the interaction of Folin-Ciocalteau reagent with phenolic side chains and peptide bonds in the protein molecule.

(b) ATPase activity

ATPase activity was measured by a procedure of Schoner *et al.* (125) which provided a system for the regeneration of substrate ATP according to the reaction sequence illustrated in Fig. 7. The conversion of NADH to NAD was followed at 340 nm and recorded by a Gilford-2400 spectrophotometer fitted with an automatic cuvette changer and temperature controlled sample chamber. The molar extinction coefficient of NADH in water is $6.22 \times 10^{+3}$/mole/cm^2. The enzyme activity was assayed at 37°C.

The assay medium contained 3.1 mM phosphoenol pyruvate (disodium salt), 22 U/ml pyruvate kinase (rabbit muscle), 11 U/ml lactic dehydrogenase (rabbit muscle), 0.017 mM NADH (disodium salt), 1.5 mM ATP (disodium salt), and 6-50 ug protein/ml of the enzyme preparation. The buffer contained 50 mM glycylglycine, 1 mM $MgSO_4$, and 0.1 mM Na_2H_2EDTA, 40 mM NaCl, 10 mM KCl, 125 mM sucrose adjusted to pH 7.6 with Tris (Tris hydroxymethylamino methane). The concentration of ATP was determined spectrophotometrically at 260 nm at pH 7.6. The extinction coefficient of ATP is $15.4 \times 10^{+3}$/mole/cm^2. Ouabain-insensitive-Mg^{++}-ATPase activity was measured in the presence of 0.4 mM ouabain.

After temperature equilibration, the reaction was started by adding 10 or 20 ul of enzyme suspension to 3 ml of the medium immediately followed by 50 ul of 90 mM ATP. An example of the trace recorded by the Gilford

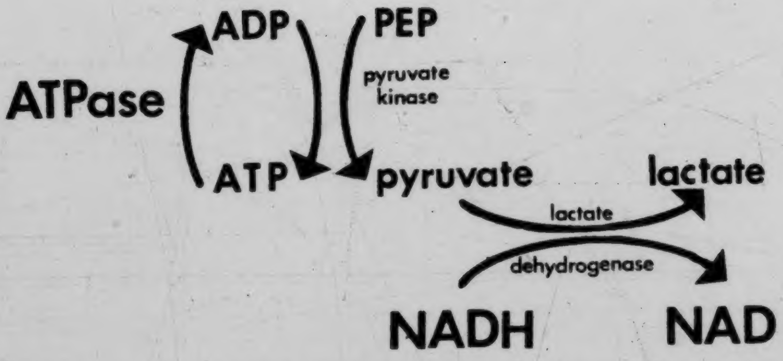


Fig. 7 Reaction sequence which is the basis of the ATPase assay taken from Mahler and Cordes. (Full details can be found in Reference No. 83).

instrument is presented in Fig. 8. Enzyme activities were calculated from the slopes of the traces and expressed as umoles ATP hydrolyzed/ mg protein/hr.

(c) *p*-NPPase activity

In most experiments *p*-NPPase activity was assayed optically by following the conversion of *para*-nitrophenyl phosphate (*p*-NP) at 400 nm (Fig. 9). Basal *p*-NPPase activity was measured in a medium containing 50 mM glycylglycine, 50 mM $MgSO_4$, and 0.5 mM Na_2H_2EDTA, adjusted to pH 7.6 with Tris; total *p*-NPPase activity was measured in the presence of 10 mM KCl; and ouabain-insensitive *p*-NPPase activity was measured in the presence of 10 mM KCl plus 2 mM ouabain.

In some earlier experiments a procedure was followed which was adapted from Izumi *et al.* (68), Tashima and Hasewaga (148), Albers and Koyal (4), and Bergmeyer (21). Here, *p*-NPPase activity was assayed in 0.5 ml medium in 5 ml test tubes in a Dubnoff metabolic shaking incubator. The reaction was terminated by adding 3.0 ml of 0.1 N NaOH, and the amount of *p*-NP produced was measured spectrophotometrically at 400 nm using a Gilford 300-N micro-sample spectrophotometer.

Later the assay procedure was adapted to the Gilford-2400 instrument where the reaction could be monitored continuously, thus eliminating the need to terminate the reaction with NaOH. Initial rates of the reaction could be calculated from the initial slopes of the recordings (Fig. 10). The activities were expressed as umoles *p*-NP produced/mg protein/hr. Potassium-stimulated activity (K^+ - *p*-NPPase) was calculated by subtracting basal *p*-NPPase activity from total *p*-NPPase activity; ouabain-inhibitable activity (O.I.-*p*-NPPase) was calculated by subtracting the ouabain-insensitive activity from total *p*-NPPase activity.

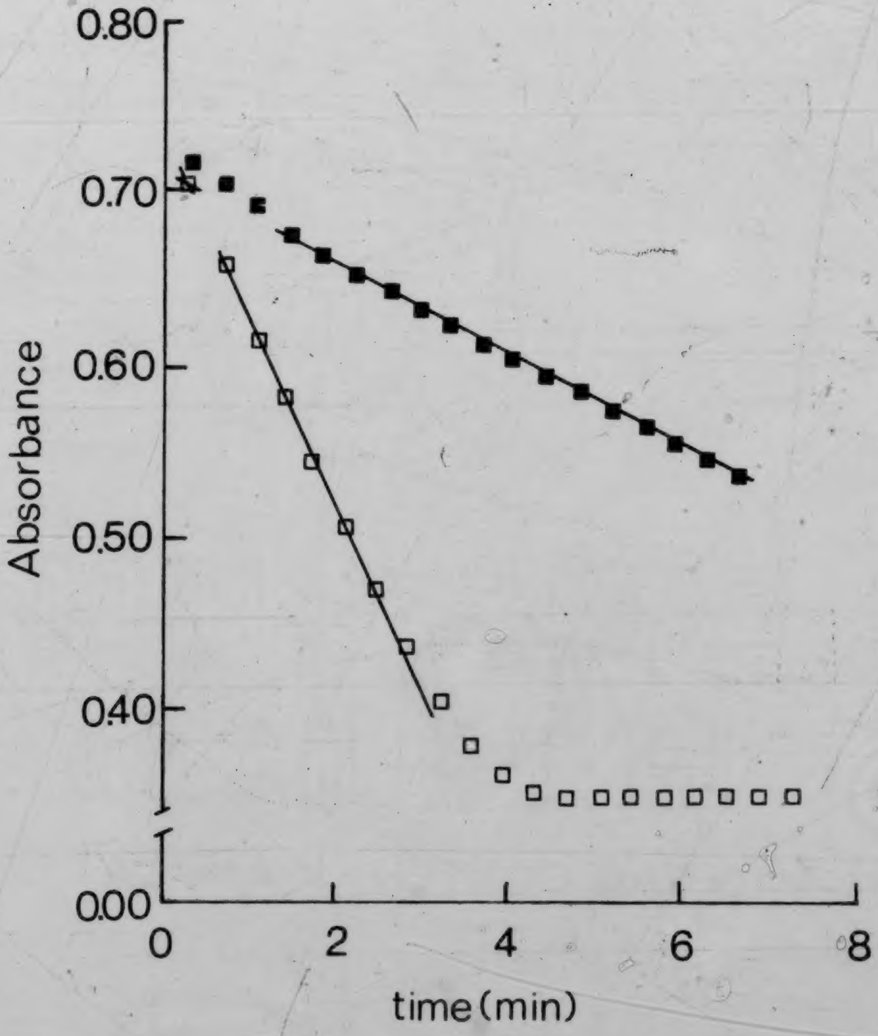
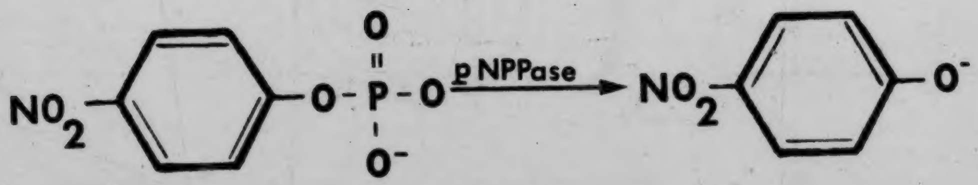


Fig. 8. A graphic representation of a typical Gilford recording of the ATPase reaction in the absence (□) and presence (■) of ouabain. (Symbols represent points automatically plotted by the Gilford instrument.)



p-NPP

p-Nitrophenyl phosphate

p-NP

p-Nitrophenol

Fig. 9. Conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol by *p*-nitrophenylphosphatase.

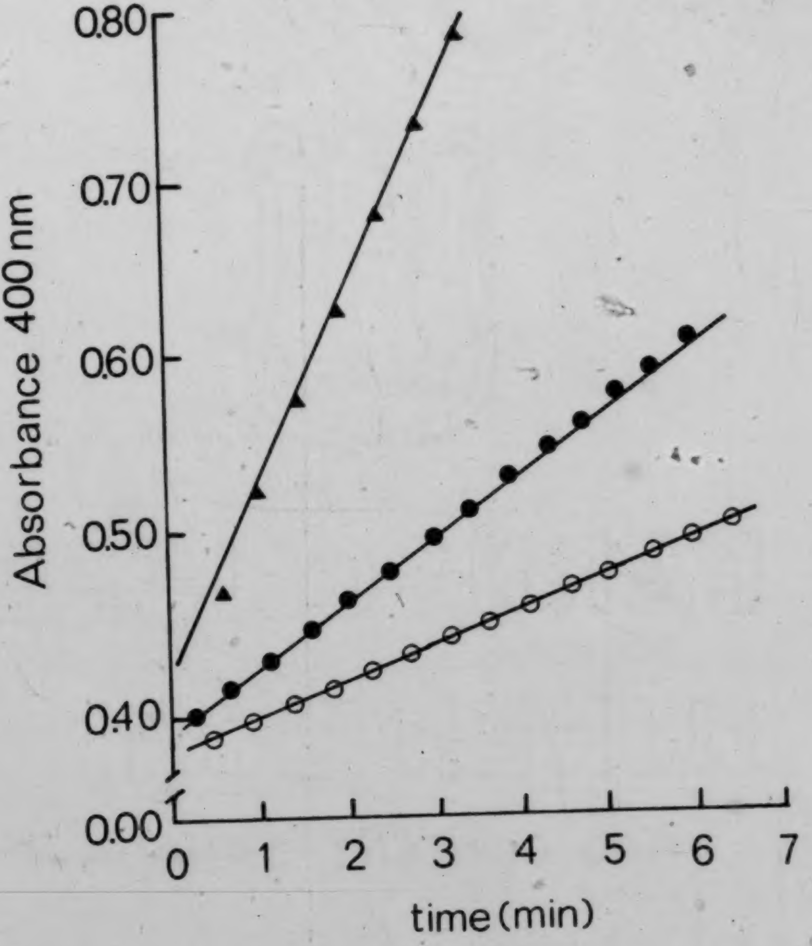


Fig. 10. Graphic representation of a Gilford recording of basal (o), total (▲), and ouabain-insensitive (●) *p*-NPPase activities.

2. Preparation of ATPase from beef brain

Frozen beef brain (*Bos taurus*) tissue was divided into 20 g aliquots which were minced with scissors, immersed in 200 ml ice-cold homogenizing solution (0.25 M sucrose, 30 mM histidine, 5 mM EDTA, pH 6.8) and homogenized in a Brinkman Polytron PT 10.20, 350 D homogenizer at 8,000 s^{-1} for 10 s. A microsomal fraction was obtained by differential centrifugation of the homogenate (Fig. 11) in a Sorvall Model RC 2-B refrigerated centrifuge fitted with an SS-34 fixed angle rotor. A microsomal enzyme suspension was prepared by suspending the final pellets ("Micro" fraction of Fig. 11) in 15-30 ml of suspending solution containing 20 mM Tris HCl, 1 mM EDTA at pH 7.6.

During the course of this work, it was found that the activity of the enzyme was greatly stabilized during storage at -20°C when sucrose was included in the suspending solution. Therefore, the suspending solution was modified to contain 0.25 M sucrose and 5 mM EDTA, at pH 7.6 (Simonson and Almeida, unpublished results).

The untreated microsomal preparation was assayed for total protein, ($Na^+ + K^+$)-ATPase activity, K^+-*p*-NPPase activity, and O.I.-*p*-NPPase activity as described above. A portion of the untreated microsomal preparation was frozen under liquid nitrogen and stored at -20°C before use, and a further portion was treated with the detergent deoxycholate (DOC).

3. Detergent treatment of ATPase

The untreated enzyme preparation (thawed immediately before use if previously frozen) was diluted to a concentration of 1 mg/protein/ml in a medium containing 6 mM Na ATP and Tris:EDTA buffer (20 mM Tris HCl, 1 mM EDTA, pH 7.6). An equal volume of 0.2% Na deoxycholate solution was added to the enzyme suspension which was then incubated at 30° for 45 min with gentle agitation in a Dubnoff metabolic shaking incubator. At the end of this

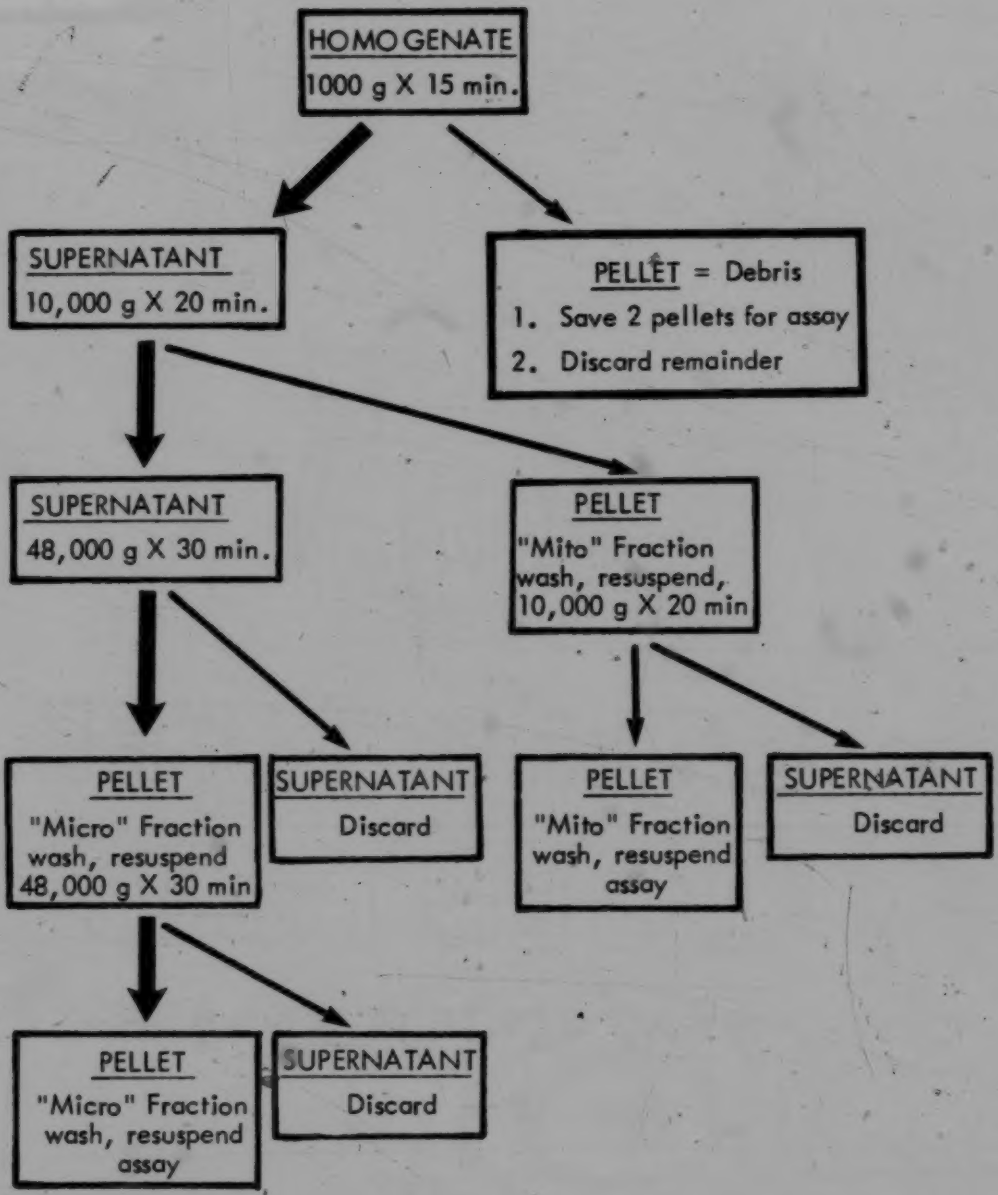


Fig. 11. Flow diagram of the procedure of differential centrifugation of beef brain membranes. Abbreviations: "Mito" = mitochondrial; "Micro" = microsomal.

treatment time, the reaction medium was centrifuged at 48,000 g for 90 min at 4°C to sediment the membrane preparation containing the enzyme activities. This was collected and washed by suspension in the sucrose-EDTA suspending solution and centrifuged again at 48,000 g for 60 min at 4°C. The particulate material was again washed and suspended as described above.

The DOC-treated membrane suspension was assayed for ouabain-inhibitable-ATPase (i.e., ($Na^+ + K^+$)-ATPase activity), and K^+- and O.I.-*p*-NPPase activities before it was frozen in liquid nitrogen and stored at -20°C. (This preparation was not frozen if it had been previously frozen and thawed.)

4. Lipase treatment of ATPase

(a) Phospholipase A (PPLA) (E.C.3.1.1.4)

A detergent-treated enzyme preparation was further treated with PPLA according to a modified version of a procedure originally described by Taniguchi and Tonomura (147). The enzyme preparation was diluted to 1 mg protein/ml in a medium described by Taniguchi *et al.* (134) which contained fat-free BSA to adsorb free fatty acids released by PPLA action. The incubation with selected concentrations of PPLA proceeded for selected periods of time at either 20° or 37°C with gentle agitation in a Dubnoff metabolic shaking incubator. A control medium not containing PPLA was always included in each treatment group.

After the activity of PPLA was terminated by chelating the calcium required for the activity with a solution of 57 mM EDTA and 321 mM Tris, the delipidated ATPase preparation was sedimented by centrifuging at 108,000 g for 90 min at 4°C in a Beckman model L3-40 refrigerated ultracentrifuge, fitted with a 60 Ti rotor. The pellet was suspended in the sucrose-containing suspending solution referred to above and centrifuged again at 108,000 g for 45 min, after which it was resuspended and assayed for total protein content and enzyme activities. This preparation was used as quickly as possible after preparation, but when necessary, was stored at 4°C to prevent further disruption of the membrane fragments by another freeze-thaw procedure.

(b) Phospholipase C (PPLC) (E.C.3.1.4.3)

PPLC treatment of untreated enzyme preparations followed the same procedure as the one described above for PPLA treatment of DOC-treated preparations except that BSA was not included in the incubation medium.

(c) PPLA plus PPLC

A DOC-treated enzyme preparation was incubated with both lipolytic enzymes in a medium containing BSA. The procedure again followed that of Taniguchi and Tonomura (147) except that the order of adding the lipases was reversed: PPLA was first allowed to react for a designated period of time before its action was terminated by adding the chelating solution; PPLC was then allowed to react for a designated time period, and was not followed by the addition of more chelating solution. The delipidated preparation was separated by centrifuging as described in section 4(a) above.

5. Effects of temperature on ($Na^+ + K^+$)-ATPase activity and on *p*-NPPase activity

(a) Arrhenius plots

The treated and untreated enzyme preparations were assayed for ATPase and *p*-NPPase activities between 8-37°C in increments of 3-4° as described in section 1 above. The initial rates of the reaction were calculated from the initial slopes of the Gilford traces and were displayed graphically as an Arrhenius plot, i.e., log of the initial rate of the reaction (log(K = Vmax)) vs. the reciprocal of the Absolute temperature ($^1/T$ (°K)).

(b) Statistical analysis

The data were analyzed by the Bogartz technique (22) for fitting a line to a cloud of points and by means of an "F test" for linearity for the points above and below a possible inflection (137). An APL/MTS computer programme was developed by Dr. D.A. Cook of the Department of Pharmacology at the University of Alberta to perform these tests and to calculate the activation energies (Ea) of the reactions over any desired temperature range.

When at least four activities were available at each temperature, a mean plot was constructed and an analysis of variance (ANOVA) was carried out using a computer programme. Mean activation energies (kcal/mole) and critical temperatures (°C) were also calculated.

Materials:-

Beef brains were provided by Gainer's Limited (Packers) of Edmonton, Alberta. ATP (disodium salt), *p*-NPP (Tris salt), $PPLA_2$ (bee venom), PPLC (*Bacillus cereus*), LDH (rabbit muscle), pyruvate kinase (rabbit muscle), phosphoenol pyruvate (disodium salt), ouabain octahydrate, bovine serum albumin (fat free), NADH (disodium salt), were all purchased from Sigma Chemical Company. DOC (disodium salt) was purchased from BDH Chemicals. All other chemicals were of reagent grade.

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RESULTS

A. Control Preparations

Before the possible effects of phospholipase treatment of potassium-stimulated-ouabain-inhibitable-*para*-nitrophenyl phosphatase (K+-, O.I.-*p*-NPPase, E.C.3.6.1.7) could be determined, it was essential to establish reference criteria. This was achieved by examining three parameters of the enzyme preparations whose values served as a basis for comparison of the effectiveness of the various lipolytic treatments in subsequent experiments. These three parameters were:

1. the Specific Activity of ($Na^+ + K^+$)-ATPase as measured by ouabain inhibition at 37°C, and the specific activity of *p*-NPPase as measured by both K^+-stimulation and by ouabain inhibition (O.I.) at 37°C, expressed as μmoles Pi produced per mg protein per hr.
2. the Ouabain Inhibition of the enzyme preparation at 37°C, i.e., the fraction of the total ATPase activity which could be inhibited by 0.4 mM ouabain and the fraction of the K^+-*p*-NPPase which could be inhibited by 2.0 mM ouabain. The ouabain-inhibitable fraction of both enzyme activities was calculated from direct measurements of the ouabain-insensitive component of the enzyme system.
3. the effects of Temperature on the ($Na^+ + K^+$)-ATPase activities and on the K^+- and O.I.-*p*-NPPase activities. By determining the temperature-activity relationship of an enzyme within the range of 8-37°C, sufficient data points (8-10) were obtained to construct an Arrhenius plot. An apparent energy (or energies) of activation (Ea) of the enzyme and the "critical temperature" (Tc) of the membrane system could be calculated from the

slope(s) of the Arrhenius plot. Ea_I and Ea_{II} designate the apparent energies of activation (in kcal/mole) of the enzyme above and below the critical temperature, respectively. The critical temperature, Tc (expressed in °C) is defined as the point of intersection or inflection of a non-linear Arrhenius plot and is believed to represent the approximate midpoint of the thermal transition of the membrane lipids (18). Henceforth, this temperature-activity relationship of an enzyme as displayed by an Arrhenius plot and as described by the values of the activation energies and of the critical temperature, will be referred to as the temperature dependence of the enzyme.

1. Optimum assay conditions

Preliminary experiments were performed to ensure that optimum concentrations of the components of the *p*-NPPase assay medium were present. Optimum concentrations were those which allowed the *p*-NPPase reaction to proceed at maximum velocity (Vmax).

The composition of the assay medium was as described in Methods, P.67. The components which were re-examined at this time were (a) substrate concentration, (b) ouabain concentration, and (c) enzyme concentration.

(a) Substrate concentration

An untreated enzyme suspension in a concentration of 171.4 ug protein/ml was reacted with 0.02 - 15.0 mM *p*-NPP at 37° and at 15°C. These selected temperatures represented points well above and below the critical temperature of the membrane lipids of these preparations as determined previously in this laboratory (32, 33) (Table 4). A plot of K^+-*p*-NPPase activity vs. substrate concentration (Fig. 12) demonstrated that a substrate concentration of 10 mM was sufficient to attain maximum enzyme activity at both temperatures without product inhibition. Therefore, 10 mM *p*-NPP was used in the assays of *p*-NPPase activity at all temperatures.

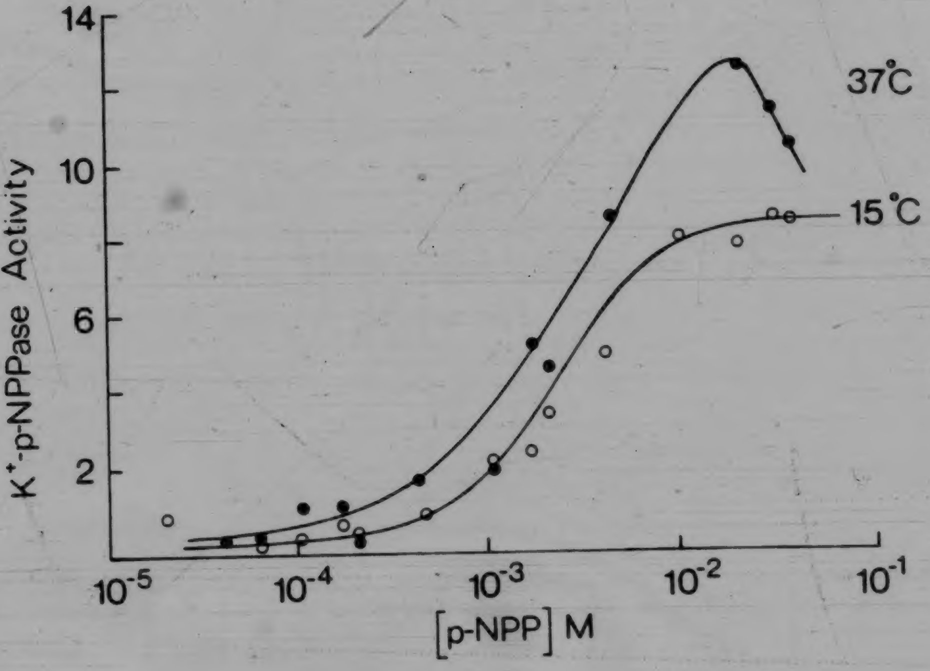

Fig. 12. The effect of substrate (*p*-NPP) concentration on K^+-*p*-NPPase activity (μmoles Pi/mg protein/hr) at 37°C (●) and at 15°C (○).

(b) Ouabain concentration

Ouabain-inhibitable *p*-NPPase activity of all the enzyme preparations was examined using 0.005 - 2.2 mM ouabain with 0.010-0.090 mg protein/ml. Dose-response curves for ouabain had already been obtained in this laboratory for the *p*-NPPase activities of untreated and DOC-treated enzyme preparations (Simonson and Charnock, unpublished results). Maximum inhibition of K^+-*p*-NPPase activity at 37°C was attained by using an excess concentration of 2.0 mM ouabain.

When dose-response curves for ouabain were obtained for the *p*-NPPase activities of the delipidated preparations, it was confirmed that 2.0 mM ouabain still maximally inhibited K^+-*p*-NPPase activity of both PPLA-treated and PPLC-treated enzyme preparations. Therefore, a concentration of 2.0 mM ouabain was used in all the assays for O.I.-*p*-NPPase activity at all temperatures. The examination of the ouabain-inhibitable-*p*-NPPase activity as well as of the ouabain-inhibitable-ATPase activity of delipidated preparations is described in greater detail below.

(c) Enzyme concentration

The *p*-NPPase activities of untreated and DOC-treated enzyme preparations were examined at 37°C using 0.034-0.572 mg protein/ml assay medium. The specific activities calculated from four different concentrations of either treated or untreated enzyme preparations were equal within experimental error (± 5%). Therefore, enzyme concentration could be varied within this range without significantly altering the values of specific activity.

2. Control values

Since both untreated and DOC-treated enzyme preparations served as substrates for lipolysis by both PPLA and PPLC, both enzyme preparations were considered as "controls". The mean values of the three parameters serving as reference criteria are listed in Table 6. The results in this table clearly demonstrate that DOC-treatment enhanced the ouabain-inhibitable-(Na^+ + K^+)-ATPase activity of untreated preparations four-fold, thereby confirming previous results obtained in this laboratory (Table 4). However, extraction with DOC increased K^+-*p*-NPPase and O.I.-*p*-NPPase activities only two-fold. The *p*-NPPase activity was about ½ the (Na^+ + K^+)-ATPase activity of an untreated enzyme preparation and about ¼ the (Na^+ + K^+)-ATPase activity of a DOC-treated enzyme preparation. The *p*-NPPase activity is a proportion of the (Na^+ + K^+)-ATPase activity, but the two activities did not maintain the same proportion after treatment with DOC.

Detergent treatment with DOC enhanced the ouabain-inhibitable fraction of both ATPase and *p*-NPPase activities. The ouabain-inhibitable fraction of the *p*-NPPase activity was still greater than the ouabain-inhibitable fraction of the ATPase activity (95% vs. 84%), although a higher concentration of ouabain was necessary to demonstrate it (2.0 mM vs. 04. mM). In this case, the parameter of ouabain inhibition changed similarly for both enzyme activities.

The temperature dependence of both (Na^+ + K^+)-ATPase and *p*-NPPase activities of the untreated preparations were similar: The mean Arrhenius plots of (Na^+ + K^+)-ATPase, K^+-*p*-NPPase, and O.I.-*p*-NPPase activities of treated and untreated preparations were non-linear with critical temperatures of about 22°C. The ratio of the activation energies, Ea_I:Ea_{II}, above and below the critical temperature (Tc) was about 0.4 for the (Na^+ + K^+)-ATPase reaction and about 0.7 for the K^+-*p*-NPPase and O.I.-*p*-NPPase reactions of the untreated enzyme preparations. This relationship persisted in the (Na^+ + K^+)-ATPase reaction and in the K^+-*p*-NPPase

TABLE 6

Specific activity, ouabain inhibition, and activation energies of untreated and DOC-treated enzyme preparations

Enzyme preparations	Mean specific activity* umoles/Pi/mg/hr	Ouabain Inhibition* %	Ea_I kcal/mole	Ea_{II}	$Ea_I:Ea_{II}$	Tc °C
Untreated						
$(Na^+ + K^+)$-ATPase	27.6 ± 2.28	79.1 ± 2.78	12.2	34.2	0.36	21
K^+-*p*-NPPase	13.7 ± 2.86	----	8.7	13.1	0.66	22
O.I.-*p*-NPPase	11.6 ± 2.30	86.1 ± 2.29	10.0	15.1	0.66	23
DOC-treated						
$(Na^+ + K^+)$-ATPase	79.6 ± 9.92	84.2 ± 3.71	13.9	34.6	0.40	22
K^+-*p*-NPPase	23.9 ± 2.33	-----	9.3	13.6	0.68	20
O.I.-*p*-NPPase	24.7 ± 1.83	97.8 ± 0.33	8.7	16.4	0.53	21

*(The values are the means ± SEM of 8 determinations.)

reaction after detergent treatment of the preparations. Ea_{II} of the ($Na^+ + K^+$)-ATPase activity was about three-fold greater than Ea_{II} of the K^+-*p*-NPPase activity and about two-fold greater than Ea_{II} of the O.I-*p*-NPPase activity (Fig. 13a, b, c). Therefore, the overall ($Na^+ + K^+$)-ATPase reaction required more energy than the *p*-NPPase reaction to proceed at the lower temperatures, and this requirement did not change after DOC treatment. The implications of these thermodynamic changes will be elaborated upon in the Discussion.

B. Phospholipase Treatments

1. Conditions of lipolysis

Conditions of lipolytic treatment were sought which would maximally reduce enzyme activities without abolishing them completely, and which would thus permit the detection of any changes in the temperature dependence of the enzyme activities. Such conditions were achieved by selecting a suitable temperature of lipolysis, a suitable phospholipase : protein ratio, and a suitable duration of lipolysis.

(a) Temperature of lipolysis

Initially, PPLA treatments were carried out at 37°C. However, L.L.M. van Deenen (personal communication) suggested that phospholipids of an erythrocyte membrane were most susceptible to lipolytic attack at their transition temperature where the change in entropy per degree is maximum. Therefore, a few PPLA treatments of our beef brain preparations were carried out at 20°C, the apparent cirtical temperature of the membrane lipids as demonstrated by the inflection point of the Arrhenius plots of the enzyme activities of control preparations. The concentration of PPLA was not altered, but the duration of lipolysis was increased to 60 min at 20°C.

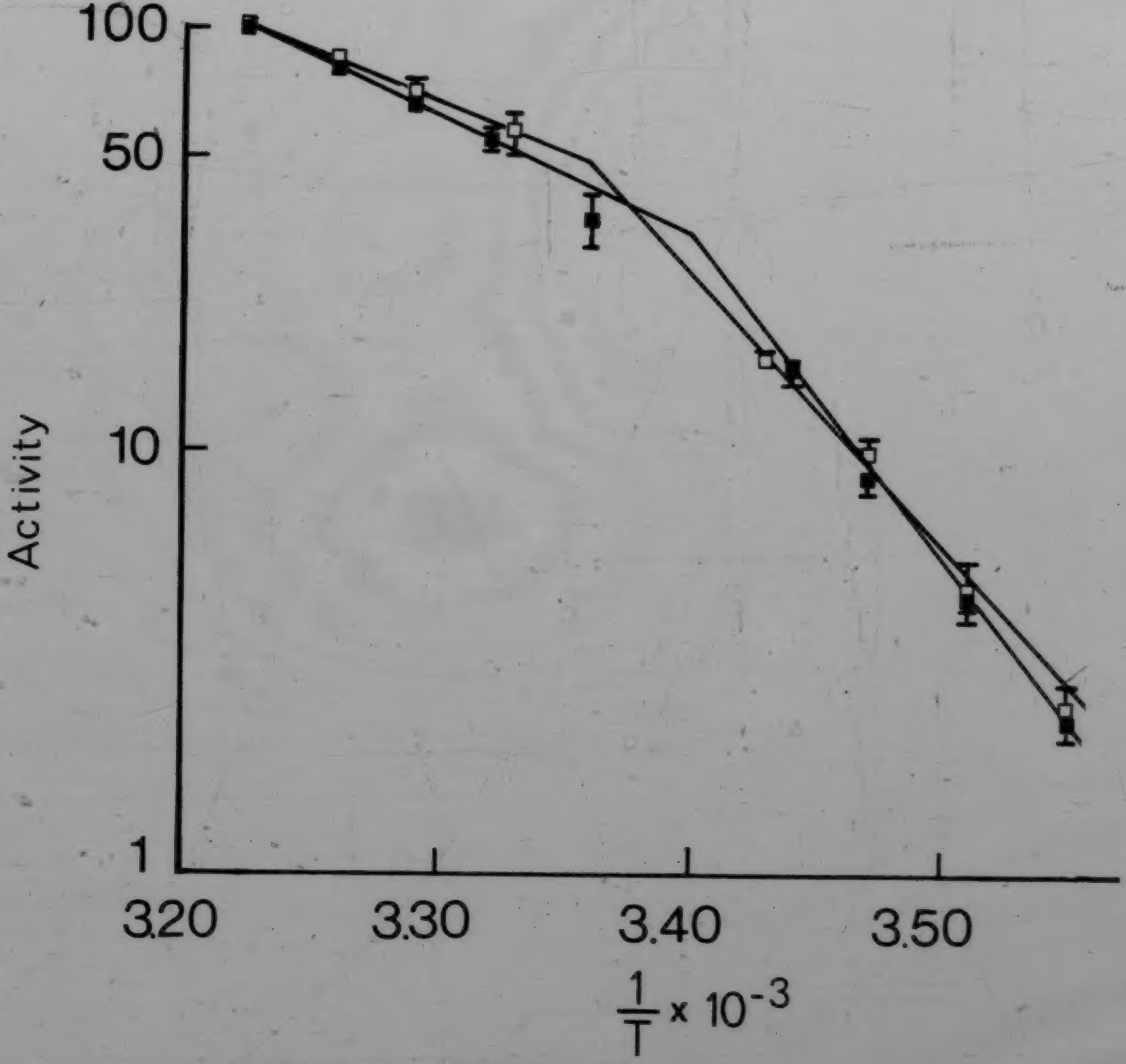

Fig. 13a Arrhenius plot of $(Na^+ + K^+)$-ATPase activity (umoles P_i/mg protein/hr) of untreated ( ) and DOC-treated ( ) enzyme preparations. Points represent the mean ± SEM of 4 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

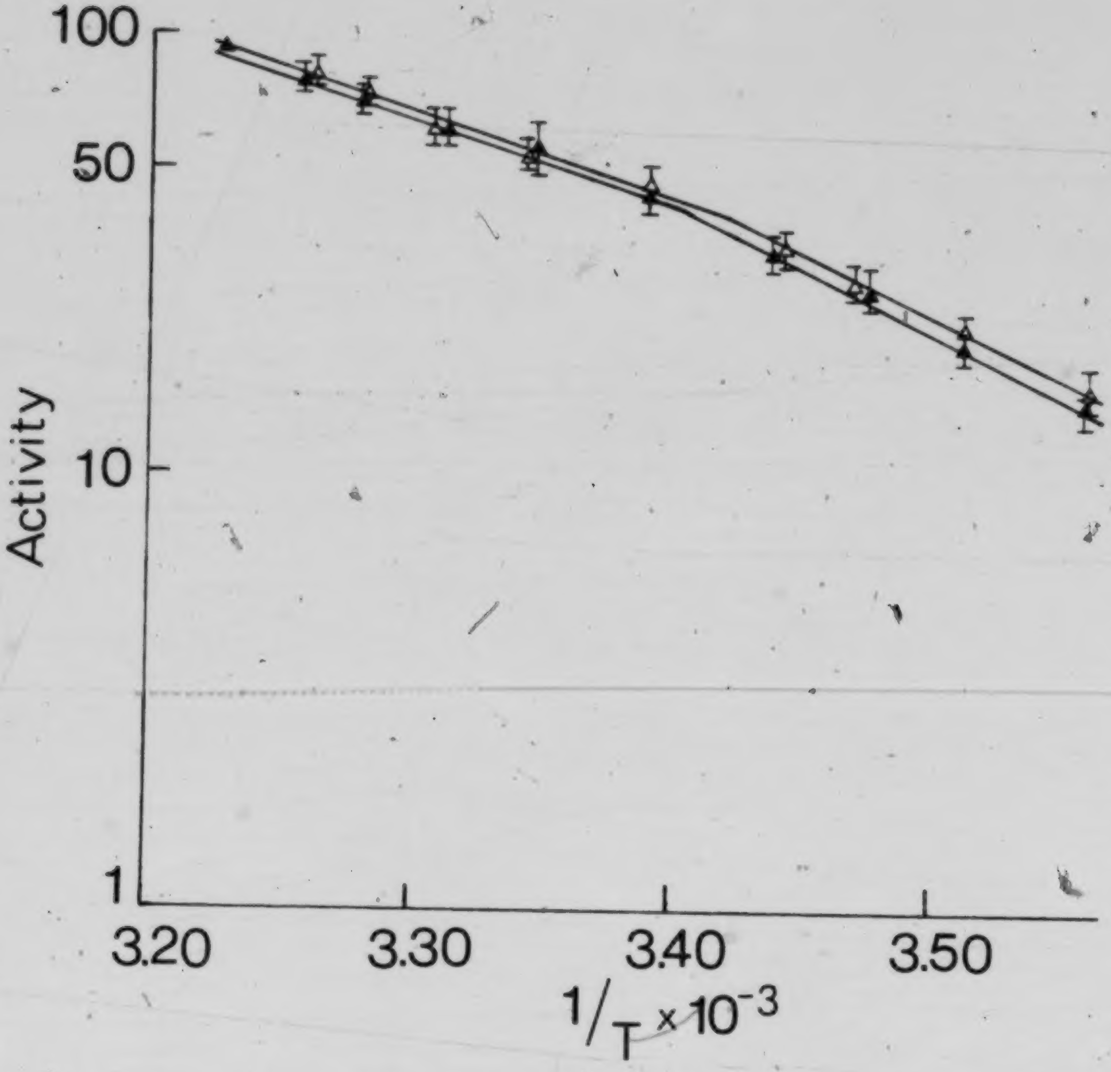

Fig. 13b Arrhenius plot of K^+-*p*-NPPase activity (μmoles P_i/mg protein/hr) of untreated (▲) and DOC-treated (△) enzyme preparations. Points represent the mean ± SEM of 8 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

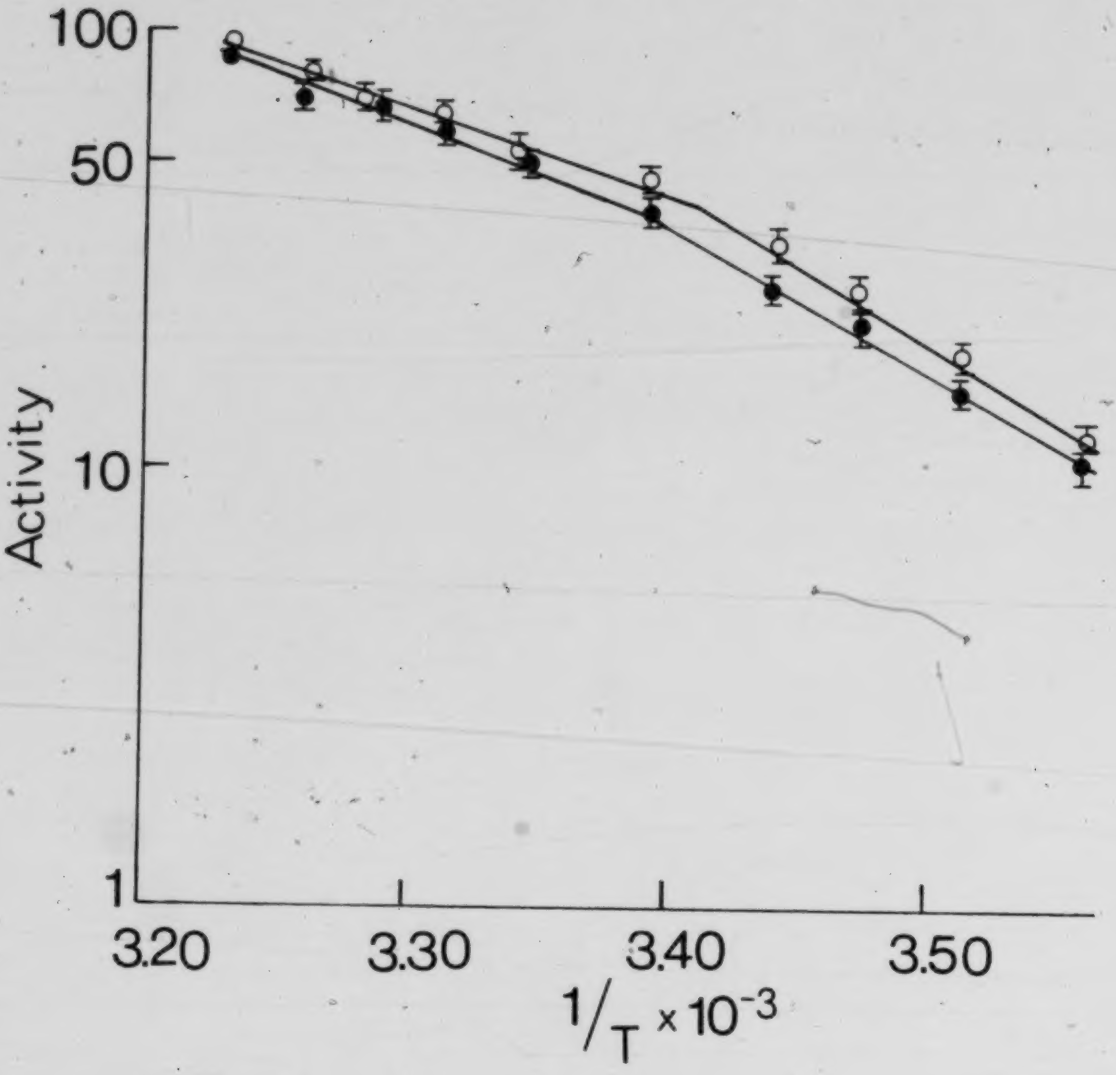

Fig. 13c Arrhenius plot of O.I.-*p*-NPPase activity (umoles P_i/mg protein/hr) of untreated (●) and DOC-treated (o) enzyme preparations. Points represent the mean ± SEM of 8 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

This procedural modification did not lower the residual enzyme activity any further than did treating with PPLA at 37°C, even though the treatment time was increased. Therefore, in subsequent incubations with PPLC (with or without PPLA), the temperature of lipolysis was not altered, and all treatments were carried out at 37°C for an appropriate period of time.

(b) Concentration of lipolytic enzymes

PPLA: Previous work had shown that incubation with 5 U PPLA/mg protein markedly reduced ($Na^+ + K^+$)-ATPase activity as well as altered its temperature dependence (32, 33). However, preliminary experiments were now carried out with 3 U PPLA/mg protein. Although this lower concentration was sufficient to significantly lower ($Na^+ + K^+$)-ATPase activity, as well as K^+- and O.I.-*p*-NPPase activities, it failed to alter the temperature dependence of any of the enzyme activities. Therefore, subsequent incubations were carried out with 5 U PPLA/mg protein at 37°C and at 20°C. This concentration markedly reduced ($Na^+ + K^+$)-ATPase activity (as previously) as well as K^+-*p*-NPPase and O.I.-*p*-NPPase activities. However, the temperature dependence of only the ($Na^+ + K^+$)-ATPase activity, but not of the K^+- nor O.I.-*p*-NPPase activities, was altered by incubation with this concentration of PPLA.

PPLC: Previous work had shown that 3 U PPLC/mg protein reduced ($Na^+ + K^+$)-ATPase activity but did not alter its temperature dependence (33). In the present situation, preliminary experiments showed that concentrations of PPLC from 3-100 U/mg protein progressively lowered the enzyme activities and produced similar changes in the temperature dependence of ($Na^+ + K^+$)-ATPase as well as of K^+- and O.I.-*p*-NPPase activities. Therefore the results were grouped together to construct mean Arrhenius plots. A concentration of 50 U PPLC/mg protein was chosen for subsequent treatments because it sufficiently lowered both enzyme activities and altered the temperature dependence of *p*-NPPase activity.

PPLA plus PPCL: When enzyme preparations were treated with both lipolytic enzymes, 5 U PPLA/mg protein and 50 U PPLC/mg protein were used.

(c) Duration of lipolysis

PPLA: Although maximum reduction of enzyme specific activities seemed to be achieved after about 8 min of incubation with PPLA at 37°C, the treatment was continued for 11 min to ensure maximum lipolytic effects under our conditions. As discussed above, at 20°C the duration of lipolysis was extended to 60 min.

PPLC: Incubations with all concentrations of PPLC tested were carried out for 60 min.

PPLA plus PPLC: When the ATPase containing membrane preparations were treated with both PPLA and PPLC, the following conditions were considered optimal: PPLA was added first and allowed to react for 11 min; then PPLC was added and allowed to react for a further 60 min.

In summary, favourable conditions for lipolytic treatment of our enzyme preparations with PPLA existed at a temperature of 37°C, with 5 U PPLA/mg protein, for 11 min. With PPLC, favourable treatment conditions existed at a temperature of 37°C, with 50 U PPLC/mg protein for 60 min. Identical conditions were used when both enzymes were used to treat the ATPase-containing membrane preparations.

(d) Control incubations

Because many membrane bound enzymes are not stable for prolonged periods of incubation at 37°C, each time an enzyme preparation was treated with a phospholipase, another aliquot served as the control. The control sample was prepared in the same manner as the treated samples except that the lipase was not included in the incubation medium. The decrease in specific activities of treated preparations with increasing incubation time was expressed as a fraction of the value of this control preparation. (The control preparation retained over 80% of the activity of the starting material, the untreated or DOC-treated preparation. The reduction in activity was attributed to the manipulations of the procedure, i.e., incubating, centrifuging and washing.)

In order to confirm that PPLC does not require Ca^{++} for its lipolytic activity as does PPLA (147), a control incubation was carried out where PPLC acted on a DOC-treated enzyme preparation in the presence of EDTA. This was necessary because, during the serial treatment with both PPLA and PPLC, the PPLA was first inactivated by chelating Ca^{++} with EDTA before PPLC was added to react in the presence of excess EDTA (and chelated Ca^{++}EDTA).

2. Specific Activity

(a) PPLA lipolysis

The decrease in specific activity after incubating a DOC-treated enzyme preparation with 3 U and 5 U PPLA/mg protein was followed at 37°C for 30 min.

$(Na^+ + K^+)$-ATPase Activity: After the DOC-treated enzyme preparation had been treated with 3 U PPLA/mg protein for 1 min, the $(Na^+ + K^+)$-ATPase activity increased beyond the 100% control value. With prolonged treatment, the activity decreased rapidly until it was about 40% of control value after 30 min treatment (not illustrated). When the detergent-treated enzyme preparation was treated with 5 U PPLA/mg protein, the $(Na^+ + K^+)$-ATPase activity decreased to about 25% of control values within the first 8-10 min of treatment and was not observed to decrease any further after 20 min treatment (Fig. 14).

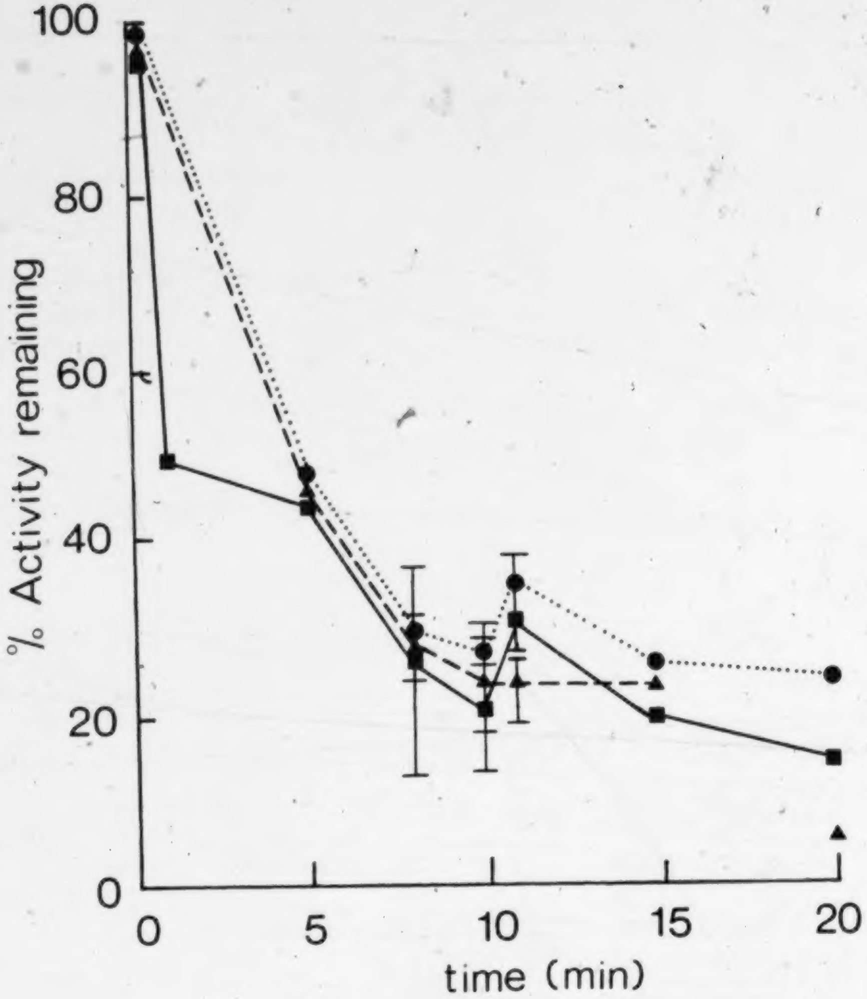

Fig. 14 Effect of time of PPLA lipolysis on specific activity. DOC-treated enzyme preparations were incubated with 5 U PPLA/mg protein at 37°C. (-■-) (Na^+ + K^+)-ATPase activity; (--▲--) K^+-*p*-NPPase activity; (··●··) O.I.-*p*-NPPase activity. (Where no error bars are shown, only one value of specific activity was available at that treatment time.)

K^+- and O.I.-*p*-NPPase Activity: After the detergent-treated enzyme preparation had been treated with 3 U PPLA/mg protein for 1 min, the *p*-NPPase activity, also, increased beyond 100% then decreased to about 20% of control after 30 min incubation (not illustrated). When the detergent-treated enzyme preparation was treated with 5 U PPLA/mg protein, the *p*-NPPase activity decreased in the same manner as did the (Na^+ + K^+)-ATPase activity, i.e., *p*-NPPase activity decreased to 25% of control values within the first 8-10 min of treatment and remained so after 20 min treatment (Fig. 14).

When the preparations were treated at 20°C, the same behaviour was seen: i.e., all three specific activities declined rapidly at first, to about 40% of control values, then more gradually to 25% of control by 40 min of incubation (Fig. 15).

To determine if ATP offered any protection against the loss of enzyme activities by PPLA action, some preparations treated at 37°C for 5 min and at 20°C for 20 min contained 4 mM ATP in the incubation medium. The resulting specific activities of these preparations were not significantly different from those of preparations treated in the absence of ATP. These results suggested that ATP did not offer any protection against reduction in activity due to lipolysis.

The results of these experiments suggested that the specific activity could not be lowered beyond 20% of control values and that the temperature dependence of the *p*-NPPase could not be altered no matter what the duration of incubation at either 37° or at 20°C. Therefore, either a certain residual amount of activity remained after lipolytic treatment irrespective of treatment conditions or the products of lipolysis hindered further lipolytic activity. The products of PPLA lipolysis were free fatty acids and lysophospholipids which should have

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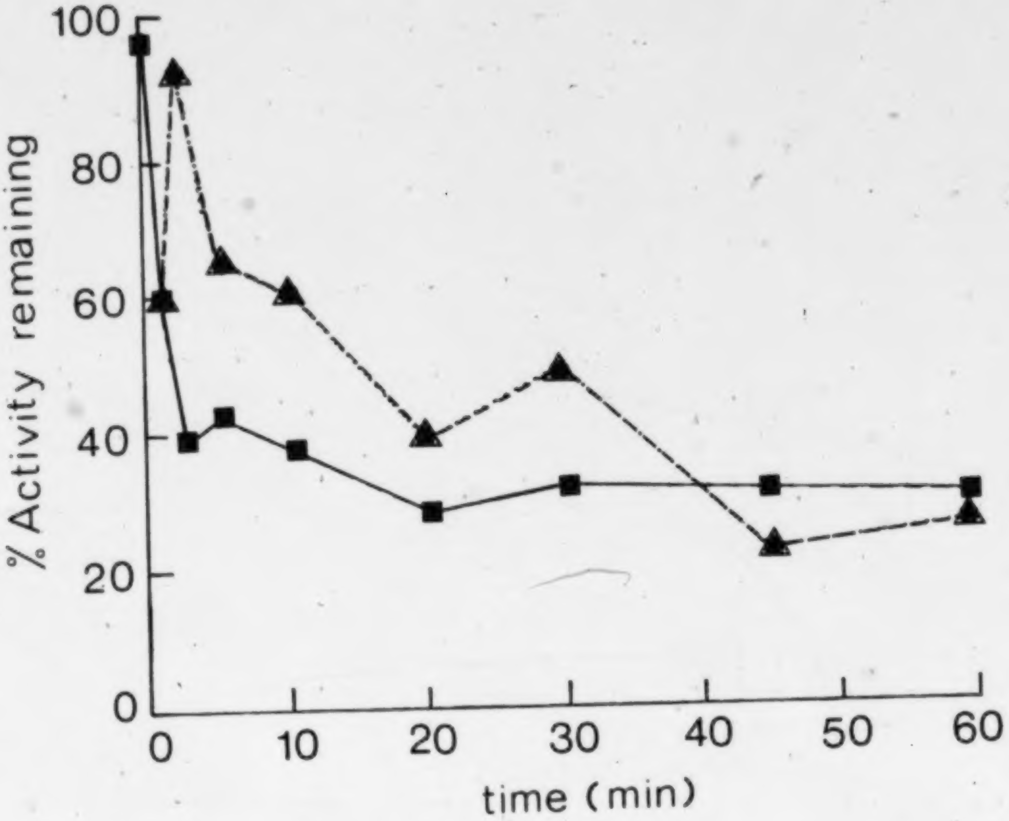

Fig. 15 Effect of time of PPLA lipolysis on specific activity. DOC-treated enzyme preparations were incubated with 5 U PPLA/mg protein at 20°C. (-■-) (Na$^+$ + K$^+$)-ATPase activity; (--▲--) K$^+$-*p*-NPPase activity; (··●··) O.I.-*p*-NPPase activity; (--▲--) and (··●··) are superimposed upon one-another.

been adsorbed by the fat-free BSA in the incubation medium. The possibility that the products of lipolysis were interfering with further lipolysis was investigated by subjecting delipidated preparations to a second lipolysis performed in two different manners at 37° and at 20°C, as described below.

At 37°C, a preparation delipidated by 5 U PPLA/mg protein for 5 min was centrifuged, washed and resuspended as described above, and then was incubated again with 5 U PPLA/mg protein for 5 min. This double-treated preparation (Table 7-A, 5 (ii)) was compared to the following three control preparations:

1. a preparation treated once for 5 min (Table 7-A, 1);
2. a preparation treated once for 10 min (Table 7-A, 2);
3. a preparation treated once for 5 min then subjected to another incubation for 5 min without PPLA in the medium (Table 7-A, 5 (i)).

The specific activities of $(Na^+ + K^+)$-ATPase, K^+-p-NPPase and O.I.-p-NPPase were further lowered by a second lipolytic treatment as were the values of the control preparation which was not treated a second time with PPLA, but was subjected to only the manipulations of a second treatment. Therefore, the further decline in enzyme activities to 6% of control values could not be attributed to PPLA action alone. In fact, as the preparation incubated a second time without PPLA in the medium is considered as a control for the double-treated preparation in the same manner as described in section B-1, (d), then, the specific activity of the double-treated preparation can be described as 100% of its control. Therefore, there was either no lipolysis, or, if some lipolysis did occur, then it did not cause a further reduction in enzyme activities. Because the second treatment did not alter either the specific activity or the temperature

TABLE 7-A

Specific activities after lipolysis with phospholipase A_2 (PPLA)

Lipolytic treatment	Specific activity (as % control)		
	$(Na^+ + K^+)$-ATPase	K^+-*p*-NPPase	O.I.-*p*-NPPase
PPLA treatment of DOC-treated enzyme preparation			
1. 5 U/mg, 5 min (3)*	24.3 ± 0.69	25.7 ± 0.41	25.7 ± 0.26
2. 5 U/mg, 11 min (6)	30.6 ± 3.39	34.8 ± 4.27	22.8 ± 3.03
3. 5 U/mg, 30 min (1)**	18.7	20.5	19.8
4. 5 U/mg, 60 min (4)**	17.4 ± 3.73	22.2 ± 1.24	21.7 ± 1.18
5. Double treatment			
i) 5 U/mg, 2 x 5 min (1)	4.2	10.4	9.3
ii) 2 (5 U/mg, 5 min) (2)	5.9 (range 5.82-6.04)	11.9 (range 8.4-15.4)	10.9 (range 7.8-14.0)
iii) 2 (5 U/mg), 60 min (1)**	14.9	17.8	17.4

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*The number of experiments is noted in parenthesis.

**All treatments were carried out at 37°C except where indicated by **: these treatments were carried out at 20°C.

dependence (described below) of the $(Na^+ + K^+)$-ATPase, K^+-*p*-NPPase, or O.I.-*p*-NPPase from those of the controls, it was not necessary to study more than one of each control preparation (Table 7-A, 5(i)) nor more than two double-treated preparations (5 (ii)). (Several temperature studies could be performed on each preparation, if considered necessary.) Three values were obtained for the specific activity of the control preparations treated once with 5 U PPLA/mg protein for 5 min at 37°C, and four values for the preparation treated once with 5 U PPLA/mg protein for 60 min at 20°C since these time periods were used in several preliminary experiments to determine optimum lipolytic treatment conditions.

At 20°C, the double treatment protocol was modified to omit the centrifuging procedure between additions of PPLA (personal communication, D.H. MacLennan). A DOC-treated enzyme preparation was incubated with 5 U PPLA/mg protein for 30 min then another 5 U PPLA/mg protein was added and the incubation was continued for a further 30 min. The delipidated preparation was collected by centrifuging as before. This double-treated preparation (Table 7A, 5 (iii)) was also compared to two control preparations:

1. a preparation treated once for 30 min (Table 7A, 3);
2. a preparation treated once for 60 min (Table 7A, 4).

Here again a second lipolytic treatment did not significantly lower $(Na^+ + K^+)$-ATPase activity and only slightly lowered K^+-*p*- and O.I.-*p*-NPPase activities from their values after a single 30 or 60 min treatment (Table 7A). The activities of all the preparations were reduced to about 20% of control. When the preparation treated for 60 min with a single addition of PPLA is considered as the control for the double-treated one, then a second treatment did not significantly lower $(Na^+ + K^+)$-ATPase activity, but lowered K^+-*p*-NPPase activity to 65% of control. The loss of activity after a second addition of PPLA at 20°C was much smaller than at 37°C, which could be due to a slower rate of lipolysis at the lower temperature. Because a drastic change was neither expected nor obtained under these conditions, only a single preparation of the 30-min control and double treated preparation was examined for specific activity. Several preparations treated for 60 min with a single addition of PPLA were available for comparison since these conditions were considered optimum for treating the enzyme preparation at 20°C.

In summary, treatment with 5 U PPLA/mg protein lowered (Na^+ + K^+)-ATPase, K^+-*p*-NPPase, and O.I.-*p*-NPPase activities to about 25% of their control values whether the enzyme preparations were treated at 37° for 10 min or at 20° for 60 min. A second treatment with PPLA did not lower the enzyme activities further. Therefore, a residual amount of enzyme activity seems to be resistant to inactivation by lipolysis with PPLA. This lipolytic agent affects both enzyme activities proportionately under our various treatment conditions.

(b) PPLC lipolysis

Initially, a concentration of 3 U PPLC/mg protein was used to treat an untreated (as opposed to DOC-treated) enzyme preparation for various periods of time (at 37°C) to compare results to those previously obtained in the laboratory (32, 33) (Fig. 16a). The (Na^+ + K^+)-ATPase activity was not significantly reduced; on the contrary, it rose considerably during the first 5-10 min of treatment then decreased to about 90% of control value. The K^+-*p*-NPPase and O.I.-*p*-NPPase activities similarly decreased after the first minute of treatment, then increased during the next 10 min of treatment to 100% of control, then decreased to 70% of the control value after 60 min of treatment.

When the untreated membrane preparations were treated with increasing concentrations of PPLC (up to 100 U/mg) for 60 min at 37°C, (Na^+ + K^+)-ATPase

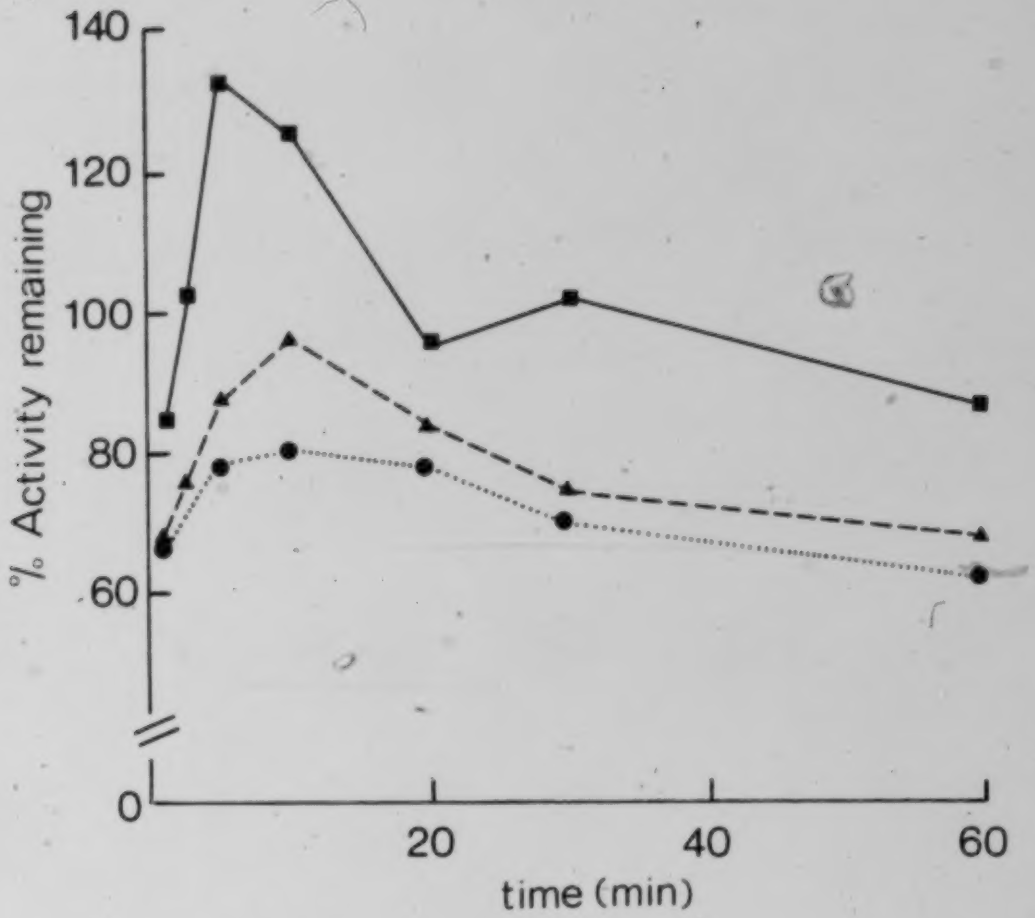

Fig. 16a Effect of time of PPLC lipolysis on specific activity. An untreated enzyme preparation was incubated with 3 U PPLC/mg protein at 37°C. (-■-) Na+ + K+)-ATPase activity; (--▲--) K+-*p*-NPPase activity; (..●..) O.I.-*p*-NPPase activity.

activity was reduced to about 20% of control, and K$^+$- and O.I.-*p*-NPPase activities were reduced to about 50% of control (Fig. 16b) with 40 or more U PPLC/mg protein.

Treatment of a DOC-treated (as opposed to an untreated) enzyme preparation with 50 U PPLC/mg protein for 60 min at 37° lowered (Na$^+$ + K$^+$)-ATPase activity to 15% of control and K$^+$- and O.I.-*p*-NPPase activities to 30-40% of control activity. When DOC-treated enzyme preparations were incubated in the presence of excess EDTA (to serve as a control for the subsequent serial treatments, described in section B, 1 (d) above), the (Na$^+$ + K$^+$)-ATPase activity was further reduced to less than 10% of control values, whereas *p*-NPPase activity was not altered from its control value. This demonstrated that PPLC was active in the absence of Ca^{++} and/or other divalent metal ions. In this case, PPLC lipolysis altered the enzyme activities disproportionately. (Table 7-B)

(c) PPLA + PPLC lipolysis

Treatment of a DOC-treated preparation with 5 U PPLA/mg protein for 11 min followed by 50 U PPLC/mg protein for 60 min lowered the (Na$^+$ + K$^+$)-ATPase activity to about 20% of the control and the K$^+$- and O.I.-*p*-NPPase activities to about 45% of control (Table 7-C). It appears that serial treatment with both lipases lowered (Na$^+$ + K$^+$)-ATPase activity to the same level as if PPLA were acting alone and *p*-NPPase activities to the same level as if PPLC were acting alone, thus suggesting some specificity of lipases for the lipids associated with these activities.

3. Ouabain inhibition

Ouabain-inhibitable-enzyme activity is defined in this thesis as that fraction of total enzyme activity which can be inhibited by ouabain at 37°C. As described in section A, 1 (b), 0.4 mM ouabain in the assay medium inhibited

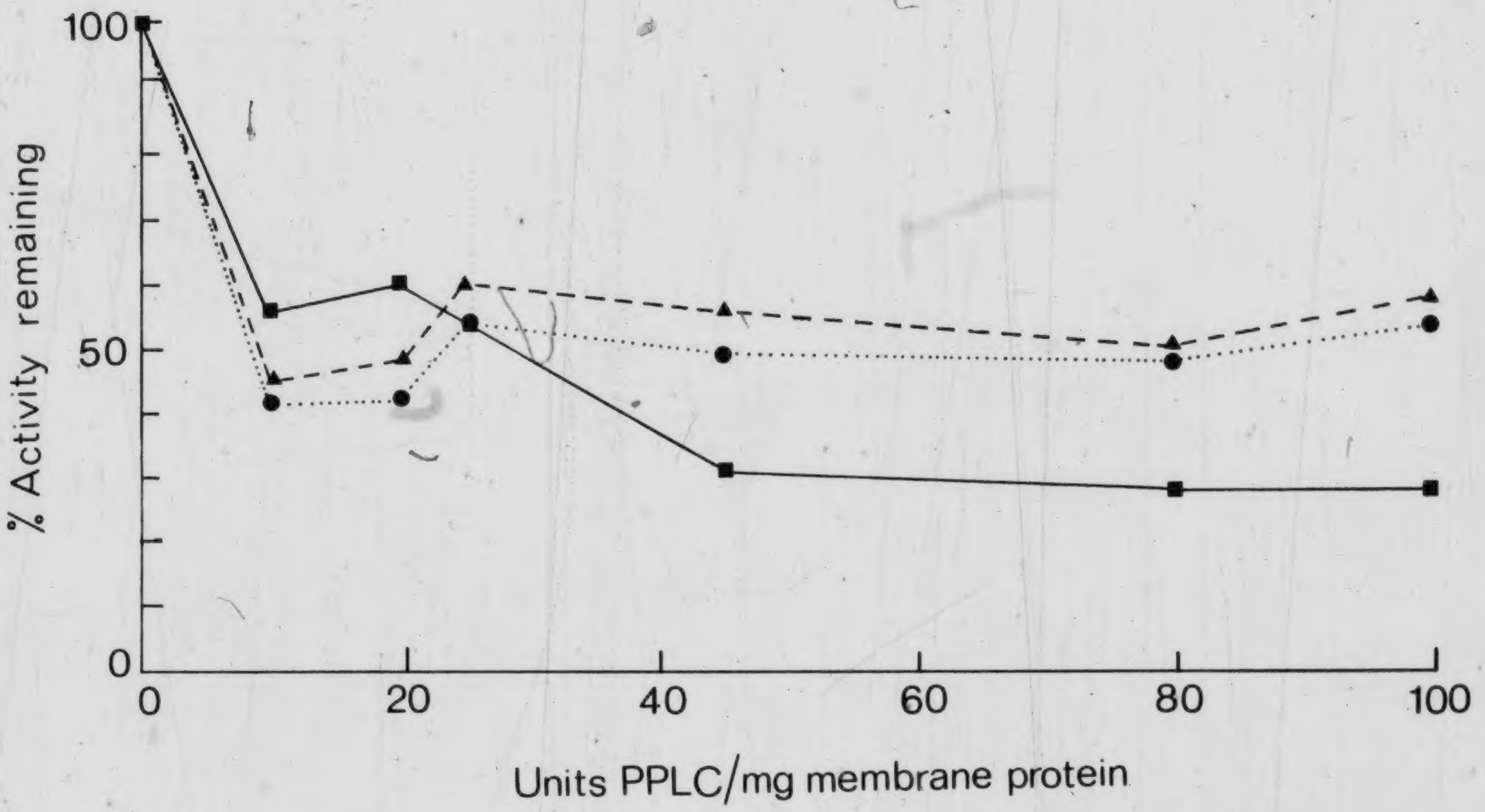

Fig. 16b Effect of PPLC concentration on specific activity. Untreated enzyme preparations were incubated with various concentrations of PPLC for 60 min at 37°C. (-■-) ($Na^+ + K^+$)-ATPase activity; (--▲--) K^+-*p*-NPPase activity; (··●··) O.I.-*p*-NPPase activity.

TABLE 7-B

Specific activities after lipolysis with phospholipase C (PPLC)

	Lipolytic treatment	Specific activity (as % control)		
		(Na$^+$+K$^+$)-ATPase	K$^+$-*p*-NPPase	O.I.-*p*-NPPase
PPLC treatment of untreated enzyme preparation at 37°C				
1.	3 U/mg, 1-60 min (7)*	85.7 – 126	75.5 ± 2.6	79.1 ± 3.91
2.	10-100 U/mg, 60 min (6)	26.6 – 69.2	50.2 ± 2.76	55.1 ± 2.82
3.	50 U/mg, 60 min (1)	69.2	55.9	53.1
PPLC treatment of DOC-treated enzyme preparation at 37°C				
4.	50 U/mg, 60 min (4)	15.0 ± 1.43	42.3 ± 1.73	38.6 ± 1.17
5.	50 U/mg, 60 min (4) (in the presence of EDTA)	7.1 ± 0.23	30.7 ± 1.80	33.1 ± 1.28

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*The number of experiments is noted in parenthesis.

TABLE 7-C

Specific activities after lipolysis with phospholipases A and C

Lipolytic treatment	Specific activity (as % control)		
	$(Na^+ + K^+)$-ATPase	K^+-*p*-NPPase	O.I.-*p*-NPPase
PPLA + PPLC treatment of DOC-treated enzyme preparation at 37°C			
5 U PPLA/mg, 11 min 50 U PPLC/mg, 60 min (4)*	19.9 ± 2.55	43.6 ± 5.47	45.3 ± 6.46

*The number of experiments is noted in parenthesis.

ATPase activity by 75% and 2.0 mM ouabain inhibited K^+-*p*-NPPase activity by 95%. As described in section A-2 above, mild lipolysis with the detergent DOC, enhanced the ouabain inhibitable portion of only ATPase. However, after lipolysis with PPLA and/or PPLC, the proportion of ouabain-inhibitable-ATPase and ouabain-inhibitable-*p*-NPPase activities was maintained. These results will be elaborated upon in section 3 (c) below. In all cases, a greater proportion of the *p*-NPPase activity than the ATPase activity could be inhibited by ouabain, although a higher concentration was necessary to do so. The implications of this concentration effect will be considered in the Discussion.

(a) Inhibitory concentrations

Ouabain dose-response curves were obtained for both the ATPase and *p*-NPPase activities of PPLA and PPLC treated enzyme preparations at 37°C. These experiments confirmed that the concentrations of ouabain used in the respective assay systems maximally inhibited the enzyme activities of preparations delipidated by the lipases (Fig. 17).

These experiments were carried out using the Gilford-2400 recording spectrophotometer. Therefore, any change in the rate of *p*-NPPase activity could be followed along with the change in the amount of ouabain inhibition. A time-dependent delay in the amount of ouabain inhibition of K^+-*p*-NPPase activity was noted at concentrations below 0.01 mM ouabain (Fig. 18). At higher ouabain concentrations, full inhibition was observed immediately. Ouabain-inhibitable-*p*-NPPase activity was calculated from the steady-state rate of the reaction which was achieved after maximal ouabain inhibition had occurred.(i.e., presumably after an equilibrium rate of ouabain binding had been reached). The time to achieve steady-state is most obvious with a ouabain concentration of 0.005 mM (Fig. 18 -●-). Here, the slope of the latter portion of the curve is obviously different than the slope of the

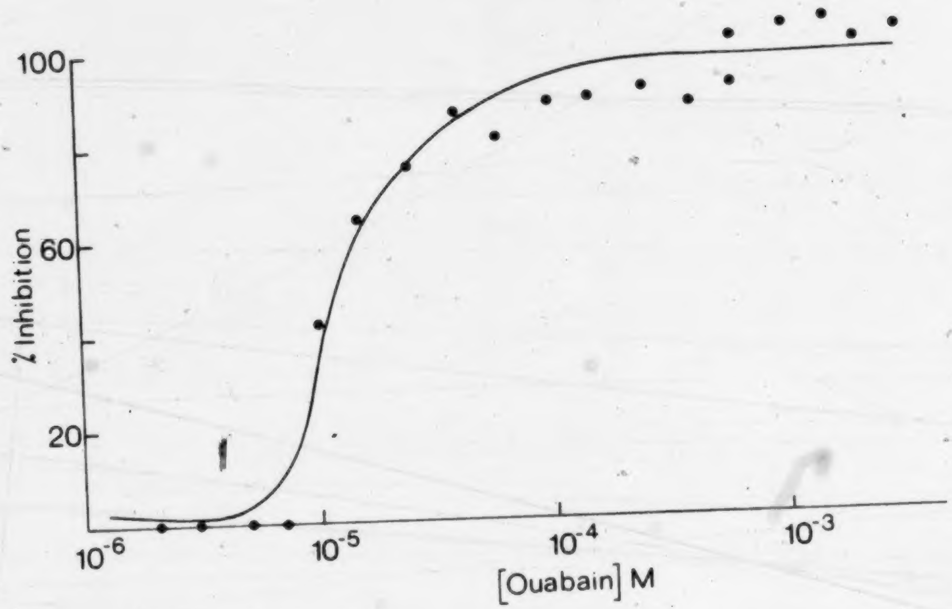

Fig. 17 Dose-response curve of ouabain inhibition of K^+-*p*-NPPase activity (steady-state levels *p*-NPPase activities were used to calculate % inhibition).

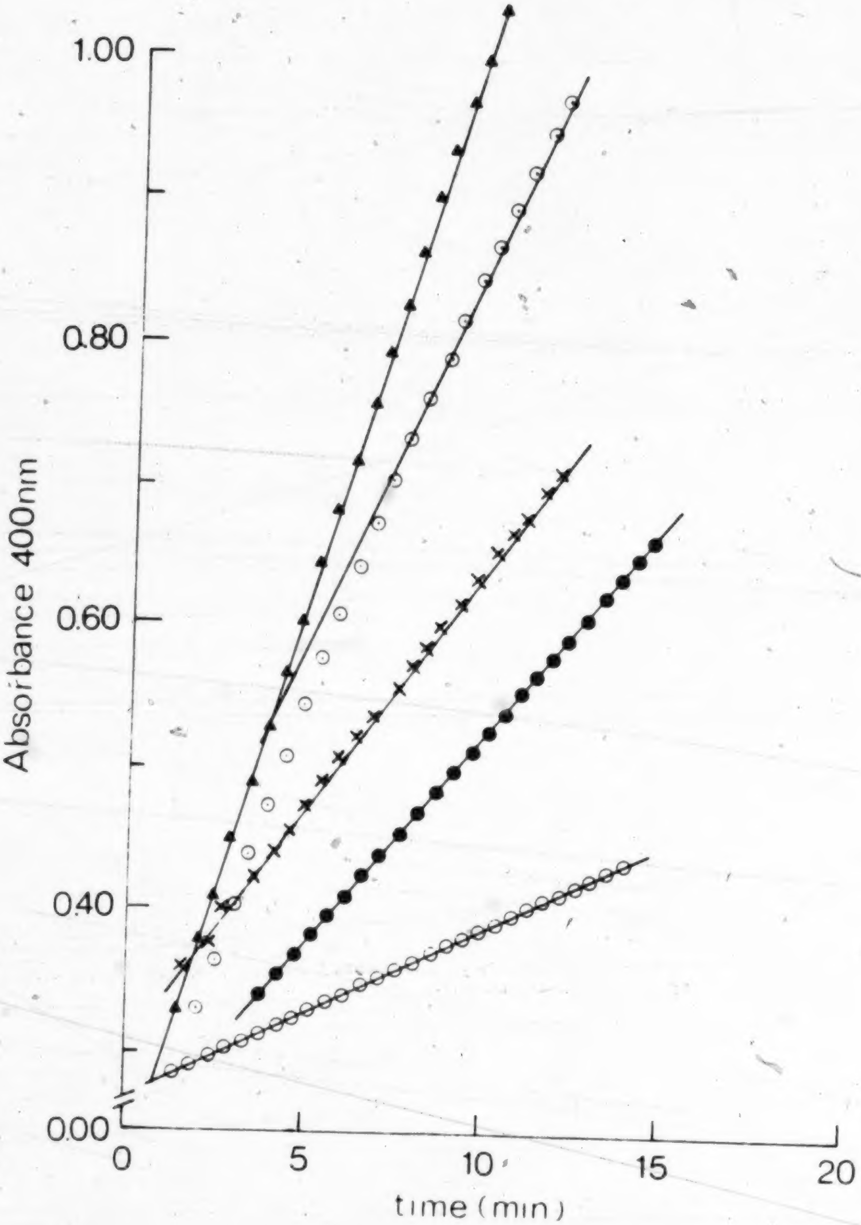

Fig. 13. *p*-NPPase reaction in the presence of various ouabain concentrations. Symbols represent points plotted by the Gilford 2400 recording spectrophotometer. (o) Basal *p*-NPPase activity (no K+, no ouabain); (▲) total *p*-NPPase activity (10 mM K+, no ouabain); (⊙) *p*-NPPase activity in the presence of 10 mM K+, 0.005 mM ouabain; (x) *p*-NPPase activity in the presence of 10 mM K+, 0.6 mM ouabain; (●) *p*-NPPase activity in the presence of 10 mM K+, 2.0 mM ouabain.

earlier portion of the curve which represents *p*-NPPase activity before the equilibrium rate of ouabain binding had been reached. The degree of ouabain inhibition was calculated from these steady-state rates and was used in constructing the dose-response curves.

(b) Temperature dependence

Previous results obtained in our laboratory had already demonstrated that the rate of ouabain binding and the fraction of ouabain-inhibitable ATPase activity increased with temperature (33, 35). This gradual increase in ouabain-inhibitable-ATPase activity was confirmed in the present study and was found to occur in the *p*-NPPase activity also. This temperature dependent increase in ouabain inhibitable enzyme activities is shown in Tables 8 (A-D). Although the maximum amount of ouabain-inhibitable-($Na^+ + K^+$)-ATPase activity was 75% of total ATPase activity at 37°C, these values have been set at 100% and the values of ouabain-inhibitable-($Na^+ + K^+$)-ATPase activity at each temperature have been expressed relative to this maximum. For comparison, the values for ouabain inhibitable-K^+-*p*-NPPase activity have also been expressed as percentage changes from the control at 37°C.

Above 20°C, the K^+-*p*-NPPase activity can be almost completely inhibited by ouabain. The diminished ouabain-inhibitable-*p*-NPPase activity at lower temperatures was assumed to be due to a lower rate of ouabain binding, as it was with the ATPase activity (33, 35). This was confirmed when the K^+-*p*-NPPase activity was shown to be almost 100% ouabain-inhibitable when it was measured at 4°, 8°, and 10°C after the enzyme preparation had been pre-incubated with the drug for 10 min at 37°C. Results of this experiment are presented in Table 9. Because the dissociation of ouabain from the enzyme is slower at low temperatures (7, 33), most of the ouabain is expected to remain bound to the enzyme for the duration of the experiments, as the

TABLE 8-A

Ouabain inhibitable ATPase and *p*-NPPase activities of an untreated enzyme preparation

Temperature °C	% O.I.-ATPase (=(Na$^+$+K$^+$)-ATPase)	% O.I.-*p*-NPPase
37	100	100
34	97.2	99.7
31	98.0	96.5
28	100	97.9
25	85.4	90.9
22	----	91.4
18	79.1	87.8
15	69.0	88.1
12	45.2	79.7
8	38.4	65.1

TABLE 8-B

Ouabain inhibitable ATPase and *p*-NPPase activities of a detergent-treated enzyme preparation.

Temperature °C	% O.I.-ATPase (=(Na^++K^+)-ATPase)	% O.I.-*p*-NPPase
37	100	100
34	99.6	100
31	96.9	99.1
28	93.3	97.0
25	87.8	95.9
22	----	95.1
18	80.4	92.7
15	72.3	86.9
12	62.1	83.4
8	54.9	68.0

TABLE 8-C

Ouabain inhibitable ATPase and *p*-NPPase activities of a PPLA-treated enzyme preparation.

Temperature	% O.I.-ATPase (=(Na$^+$+K$^+$)-ATPase)	% O.I.-*p*-NPPase
37	100	100
34	100	94.5
31	85.3	99.3
28	91.7	100
25	51.4	95.4
22	----	91.4
18	63.2	81.5
15	64.4	77.1
12	77.2	66.6
8	38.5	46.1

TABLE 8-D

Ouabain inhibitable ATPase and *p*-NPPase activities of a PPLC-treated enzyme preparation.

Temperature	% O.I.-ATPase (=(Na^++K^+)-ATPase)	% O.I.-*p*-NPPase
37	100	100
34	102	97.7
31	92	97.4
28	93.8	96.2
25	103	97.2
22	111	90.6
18	85.5	95.8
15	79.3	76.8
12	81.0	80.8
8	81.7	56.2

TABLE 9

Change in O.I.-*p*-NPPase activities at low temperatures after preincubation with ouabain*

Temperature of reaction °C	Conditions	K^+-*p*-NPPase activity umole/mg/hr	O.I.-*p*-NPPase activity umole/mg/hr	% increase in O.I.-*p*-NPPase (after preincubation)	% Quabain inhibition
4	control	0.6 ± 0.07	0.39 ± 0.08	74.4	63.6 ± 4.87
	preincubated	0.7 ± 0.02	0.68 ± 0.04		97.5 ± 7.18
8	control	1.05 ± 0.03	0.76 ± 0.04	5.3	72.4 ± 3.61
	preincubated	0.90 ± 0.00	0.80 ± 0.02		89.0 ± 1.92
10	control	1.21 ± 0.01	0.92 ± 0.06	18.5	76.4 ± 5.30
	preincubated	1.11 ± 0.03	1.09 ± 0.05		97.9 ± 3.31

*An untreated enzyme preparation was preincubated with ouabain at 37°C for 10 min before *p*-NPPase activity was assayed at the lower temperatures indicated.

temperature is lowered. Table 9 demonstrates that ouabain must indeed remain bound to the enzyme in order to effect greater inhibition at the lower temperatures. However, these results are in contrast with previous findings with ($Na^+ + K^+$)-ATPase. When the enzyme preparation was pre-incubated with ouabain at 37°C, then assayed for ATPase activity at temperatures below 15°C, the fraction of ouabain-inhibitable-($Na^+ + K^+$)-ATPase did not approach the maximum value determined at 37°C. Except for a marginal 15% increase in the % ouabain inhibition below 12°C, this treatment did not appreciably change the % ouabain inhibition from that which was attained when the enzyme was not pre-incubated with ouabain (L.P. Simonson, unpublished results). These differences suggest a different quality of ouabain inhibition, and perhaps, ouabain binding, of these two enzyme activities. This will be elaborated upon later.

(c) Lipid treatments

Extraction with DOC or lipolysis with PPLA did not greatly alter the temperature dependence of the ouabain inhibition of either the ($Na^+ + K^+$)-ATPase reaction or of the K^+-*p*-NPPase reaction as demonstrated in Tables 8-B and 8-C. However, after PPLC-treatment, ouabain inhibition of both enzyme activities seemed to become less temperature-dependent (Table 8-D). The % ouabain inhibition at each temperature appeared to be higher than control values, even without previous pre-incubation.

The amount of ouabain-inhibitable-ATPase activity and ouabain-inhibitable-K^+-*p*-NPPase activity were not significantly altered after the various lipolytic treatments with a single addition of a lipase. A decline in the ouabain-inhibitable fraction of only ATPase activity, but not *p*-NPPase activity was noted after a double treatment or a double manipulation. e.g., treatment with a second addition of PPLA or merely the manipulations of a second treatment at 37°C lowered the % ouabain inhibition to 65.9 ± 3.24% (n = 4) and to 59.6%, respectively. Treatment with a second addition of PPLA at 20°C lowered the % ouabain inhibition to 53.6%. Treatment with both lipolytic enzymes together, (PPLA + PPLC), at 37°C lowered the % ouabain inhibition to 60.0 ± 3.18% (n = 4). These results suggested that a second treatment, with or without a lipase, may have altered a lipid component of a ouabain binding site of ($Na^+ + K^+$)-ATPase.

While monitoring *p*-NPPase activity on the Gilford instrument, the rate plots of ouabain-insensitive *p*-NPPase activity of PPLC-treated preparations became linear very rapidly at low as well as at high temperatures with or without pre-incubation of the enzyme with ouabain. Because steady-state levels of *p*-NPPase activity were attained more rapidly, these results suggested that ouabain attained an equilibrium level of binding more rapidly under these experimental conditions.

The rate of [^3H]-ouabain-binding to this PPLC-treated enzyme preparation was measured by L.P. Simonson and was found to be 3.31 pmoles/mg/sec which is about 40% greater than the value of 2.38 pmoles/mg/sec of the untreated preparation. The equilibrium level of binding increased to 186 ± 5 pmoles/mg from 129 pmoles/mg for the untreated preparation, which is also an increase of about 40% above the control. These results suggested that PPLC had indeed removed some sort of impediment to ouabain's access to its binding site(s).

Except in PPLC-treated preparations, the parameter of ouabain inhibition was not altered markedly by lipolytic treatments.

4. Temperature-activity relationships

The temperature dependence (as defined above) of the ($Na^+ + K^+$)-ATPase activities and K^+- and O.I.-*p*-NPPase activities of the phospholipase treated enzyme preparations was examined within the range of 8-37°C. The specific activity at each temperature was calculated and Arrhenius plots were constructed as described in Methods (section 6 (a)) and in the Introduction (section 7). The mean Arrhenius plots from several phospholipase treated preparations were compared to those from the untreated and DOC-treated preparations.

As described in Section A, 2 above, the mean Arrhenius plots of the $(Na^+ + K^+)$-ATPase and of the K^+-*p*-NPPase and O.I.-*p*-NPPase activities of the control preparations were non-linear and each could be described as two intersecting straight lines. The activation energies calculated from the slopes of these lines and the temperature at which the inflection occurred have already been presented in Table 6. (p. 82). To briefly recapitulate: The ratio of the activation energies above and below the inflection point (T_c) was about 0.4 for the $(Na^+ + K^+)$-ATPase reaction and about 0.7 for the K^+-*p*-NPPase and O.I.-*p*-NPPase reactions of the untreated as well as of the DOC-treated enzyme preparations. The inflection in the curves of both enzyme preparations occurred at about 20°C.

(a) PPLA lipolysis

Lipolysis with PPLA altered the temperature-activity relationship of the (Na^+ + K^+)-ATPase reaction, but not the K^+- and O.I.-*p*-NPPase reaction. As frequently demonstrated by other studies in our laboratory, the Arrhenius plot of (Na^+ + K^+)-ATPase activities was now linear. However, the plots of K^+-*p*-NPPase activities remained nonlinear and were similar to the control plots of untreated and DOC-treated enzyme preparations. When the treatment conditions were varied, i.e., temperature and duration of lipolysis, the results did not change. The mean Arrhenius plots shown in Fig. 19a and 19b are representative of the plots obtained after various durations of lipolysis at 37°C and at 20°C.

A summary of the temperature dependence of preparations treated with PPLA under various conditions is presented in Tables 10 and 11. It should be noted that treatment of the enzyme preparations twice with PPLA or subjecting them to the manipulations of a second treatment without the lipase slightly decreased the values for Ea of the ATPase activities below the values of the other delipidated preparations, but significantly increased the values for only Ea_I of the *p*-NPPase over those of other delipidated preparations

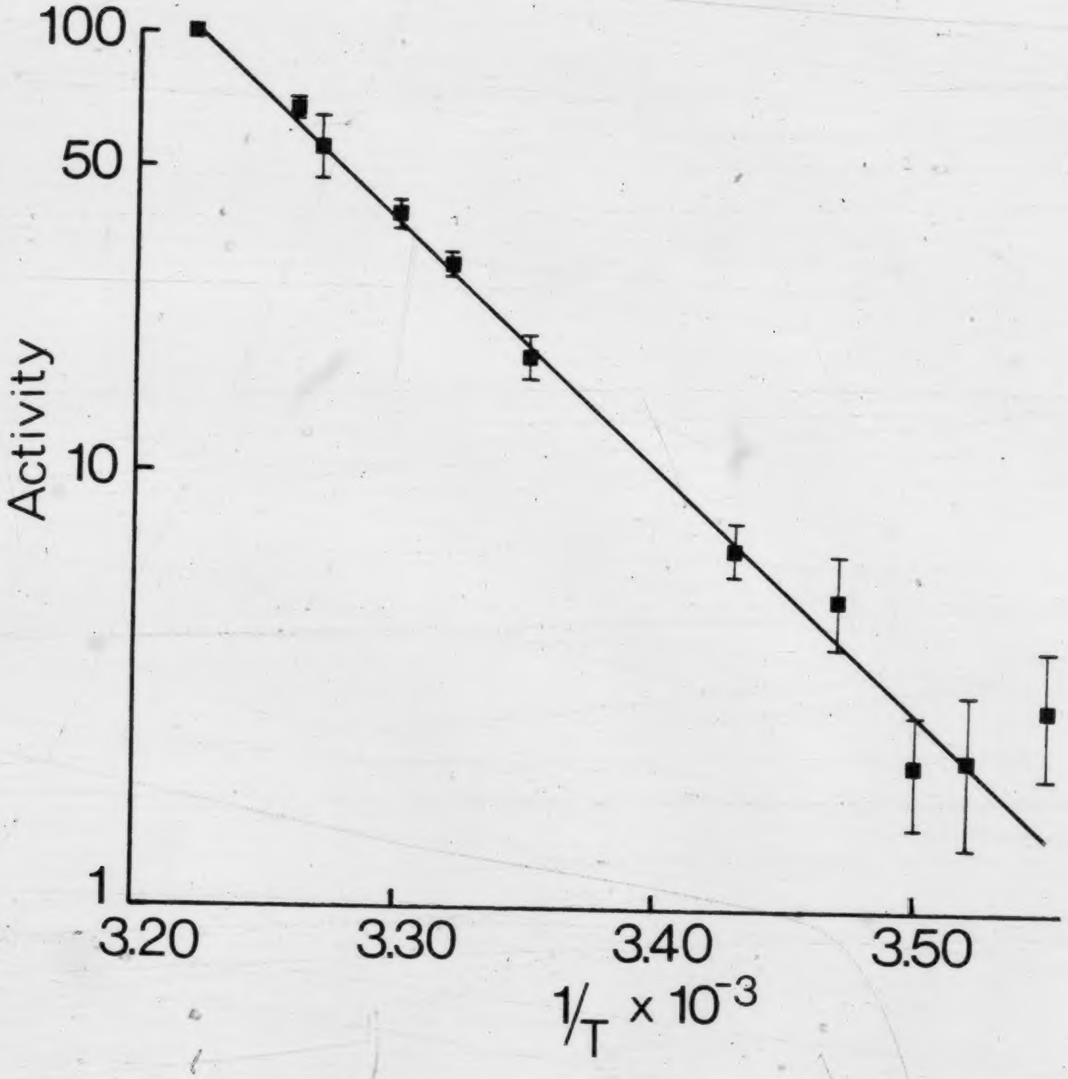

Fig. 19a Arrhenius plot of $(Na^+ + K^+)$-ATPase activity (umoles P_i/mg protein/hr) of PPLA-treated enzyme preparations. DOC-treated preparations were incubated with 5 U PPLA/mg protein for 8, 11 and 15 min. Points represent the mean ± SEM of 13 values.

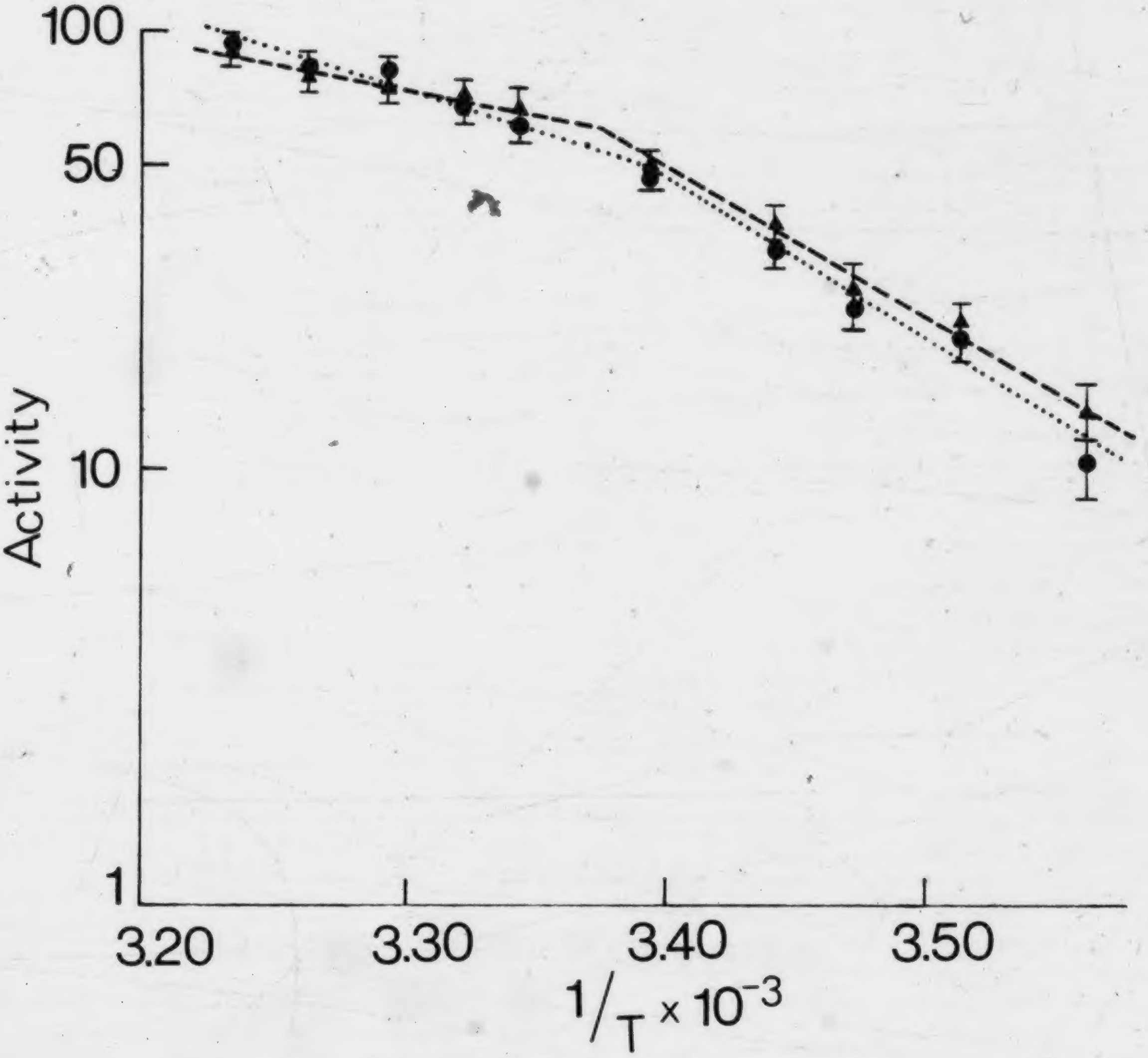

Fig. 19b Arrhenius plot of K$^+$-*p*-NPPase activity (▲) and O.I.-*p*-NPPase activity (●) of PPLA-treated enzyme preparations. Activities are expressed as umoles P_i/mg protein/hr. Treatment conditions are described in the legend to Fig. 19a. Points represent the mean ± SEM of 12 values.

TABLE 10

Temperature dependence of PPLA-treated enzyme preparations.

Treatment conditions (temperature and time)	Enzyme activity	n	Ea_I*	Ea_{II}*	Ea_I:Ea_{II}	Tc*	Remarks
37°C, 5 min	(Na^++K^+)-ATPase	3	21.3	----	----	--	linear
	K^+-*p*-NPPase	3	9.8	17.3	0.56	23	non-linear
	O.I.-*p*-NPPase	3	9.5	24.1	0.39	21	non-linear
37°C, 8, 11, 15 min	(Na^+K^+)-ATPase	13	25.2	----	----	--	linear
	K^+-*p*-NPPase	12	5.4	15.8	0.34	25	non-linear
	O.I.-*p*-NPPase	12	7.3	18.1	0.40	22	non-linear
20°C, 30 min	(Na^++K^+)-ATPase	3	26.0	----	----	--	linear
	K^+-*p*-NPPase	3	8.4	18.6	0.45	18	non-linear
20°C, 60 min	(Na^++K^+)-ATPase	1	27.4	----	----	--	linear
	K^+-*p*-NPPase	4	9.6	17.0	0.56	19	non-linear

*Values for the activation energies above (Ea_I) and below (Ea_{II}) the transition temperature (Tc, °C) are expressed as kcal/mole.

TABLE 11

Temperature dependence of PPLA "double" treated enzyme preparations

Treatment conditions (lipase concentration, temperature, time)	Enzyme activity	n	Ea_I*	Ea_{II}*	Ea_I:Ea_{II}	Tc*	Remarks
5 U PPLA/mg protein 37°C, 2 x 5 min	$(Na^+ + K^+)$-ATPase	2	19.0	----	----	--	linear
	K^+-*p*-NPPase	2	13.4	22.7	0.59	19	non-linear
	O.I.-*p*-NPPase	2	18.9	43.4	0.43	17	non-linear
2 (5 U PPLA/mg protein, 37°C, 5 min)	$(Na^+ + K^+)$-ATPase	4	16.8	----	----	--	linear
	K^+-*p*-NPPase	4	12.3	20.0	0.62	22	non-linear
	O.I.-*p*-NPPase	4	15.7	28.3	0.56	23	non-linear
2 (5 U PPLA/mg protein), 20°C, 60 min	$(Na^+ + K^+)$-ATPase	1	26.0	----	----	--	linear
	K^+-*p*-NPPase	3	8.4	17.6	0.48	21	non-linear

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*As in Table 11-A.

(Table 11). This observation reinforces the argument that the *p*-NPPase is lipid-dependent but in a manner different from the lipid dependence of the overall (Na^+ + K^+)-ATPase activity.

As stated above, previous results in this laboratory demonstrated that the Arrhenius plot of (Na^+ + K^+)-ATPase activity in PPLA-treated preparations was linear. In addition, the non-linear appearance of a control plot was restored after treatment with 3 mg phosphatidylserine/mg delipidated membrane protein (32). Because the temperature-dependence of the *p*-NPPase was not similarly altered here, such reconstitution procedures were not attempted.

(b) PPLC lipolysis

PPLC treatment of an untreated preparation did not alter the temperature dependence of (Na^+ + K^+)-ATPase (Fig. 20a). The Arrhenius plot was non-linear and could be described as two intersecting straight lines. However, the temperature dependence of the K^+- and O.I.-*p*-NPPase was altered: although the Arrhenius plot was still non-linear, it could not be adequately described as two intersecting straight lines, but it could be better described as curvilinear without the inflection previously seen at 20°C (Fig. 20b). Since any number of straight lines can be drawn through a smooth curve, it is not appropriate to calculate a single activation energy for the *p*-NPPase activity of this preparation.

PPLC treatment of a DOC-treated enzyme preparation, altered the temperature dependence of the (Na^+ + K^+)-ATPase as demonstrated by a linear Arrhenius plot (Fig. 21a). The temperature dependence of K^+-*p*-NPPase and O.I.-*p*-NPPase was, however, not altered. The Arrhenius plot was still non-linear, and could be described as two intersecting straight lines (Fig. 21b).

When PPLC-treatment of a DOC-treated enzyme preparation was carried out in the presence of EDTA, the Arrhenius plot of (Na^+ + K^+)-ATPase activity

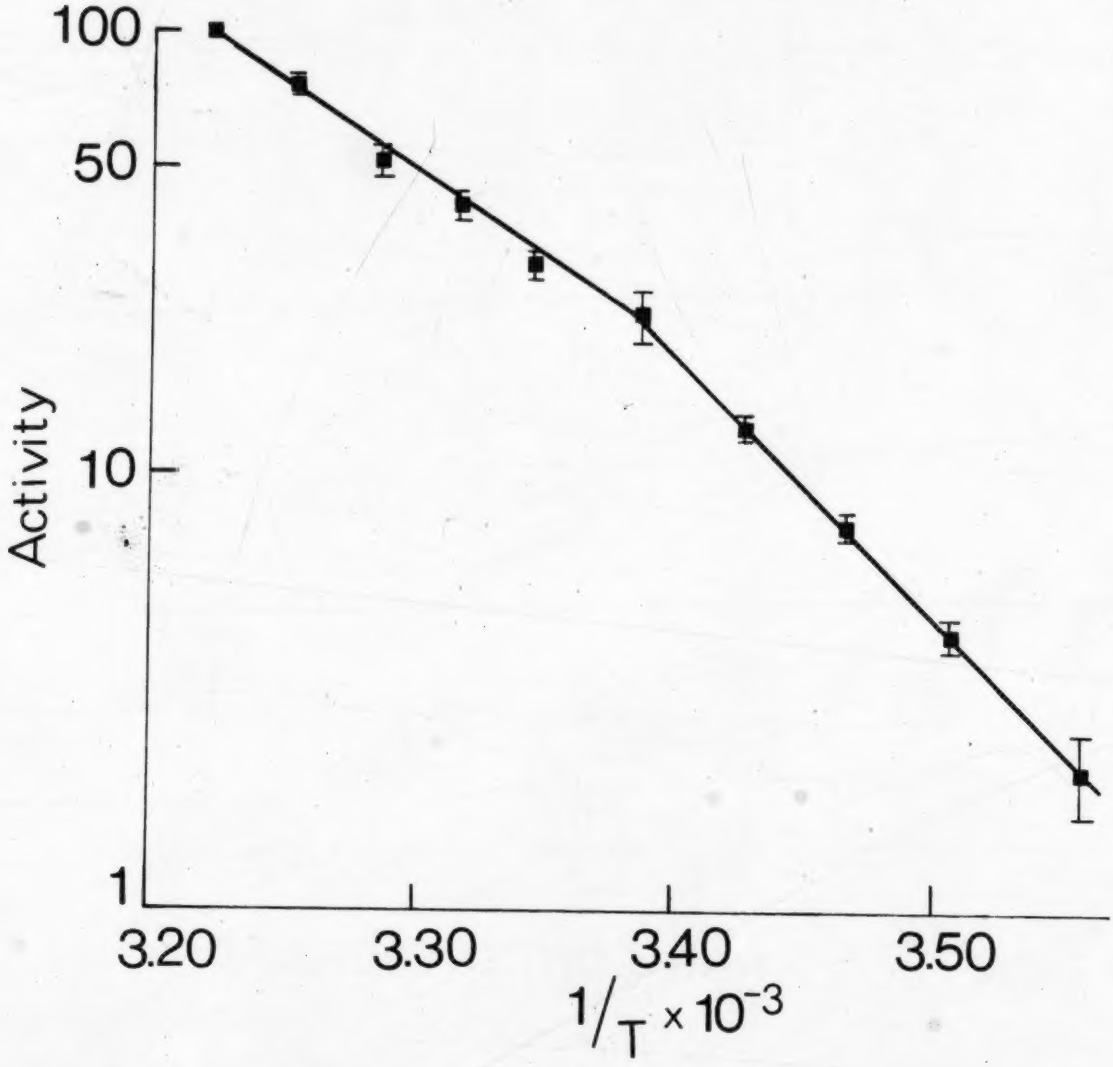

Fig. 20a Arrhenius plot of $(Na^+ + K^+)$-ATPase activity (umoles P_i/mg protein/hr) of PPLC-treated enzyme preparations. Untreated enzyme preparations were treated with 3-100 U PPLC/mg protein for 60 min at 37°C. Points represent the mean ± SEM of 5 values.

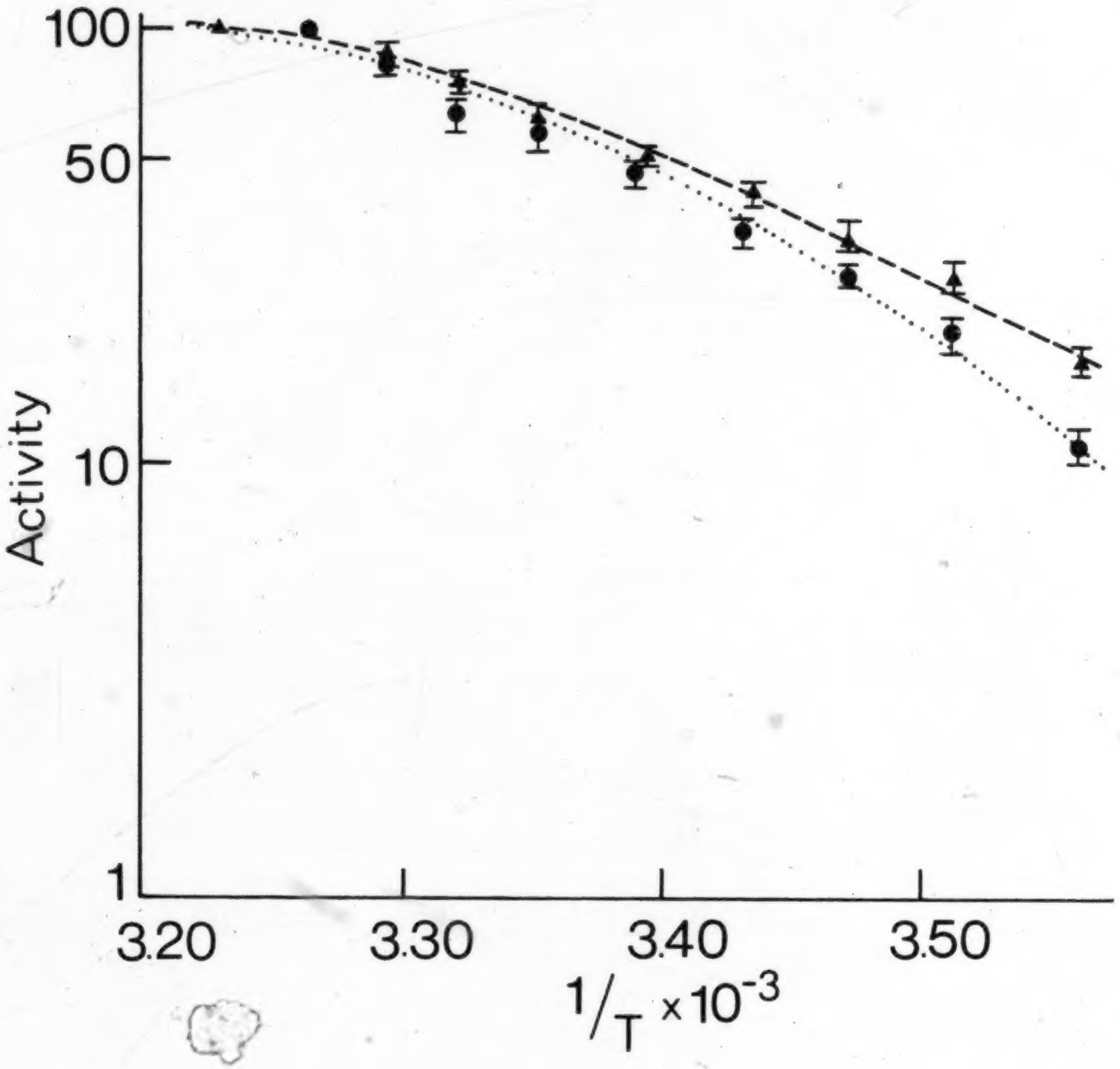

Fig. 20b Arrhenius plot of K^+-*p*-NPPase activity (▲) and O.I.-*p*-NPPase activity (●) of PPLC-treated enzyme preparations. Treatment conditions are described in the legend to Fig. 20a. Activities are expressed as umoles P_i/mg protein/hr. Points represent the mean ± SEM of 9 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

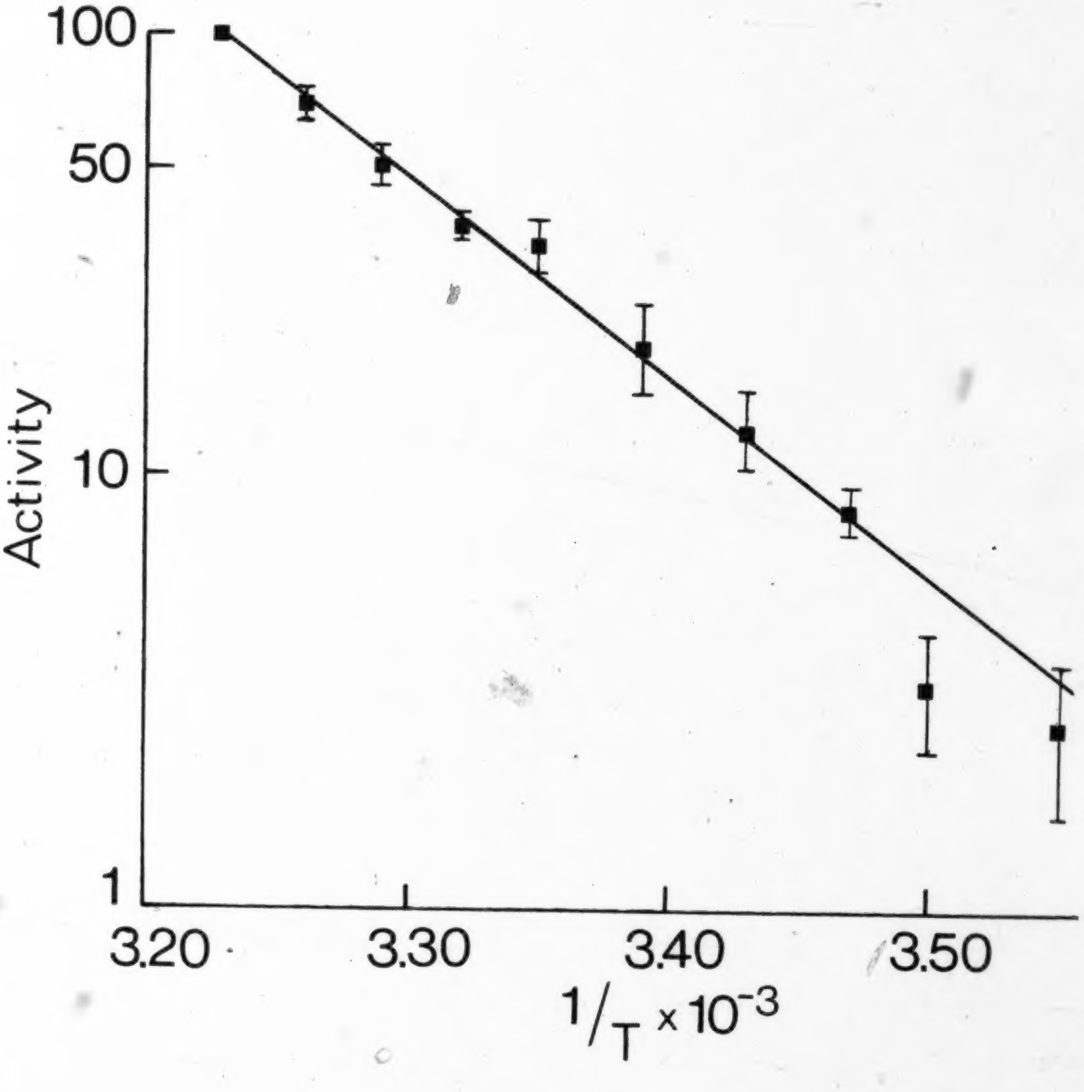

Fig. 21a Arrhenius plot of ($Na^+ + K^+$)-ATPase activity (umoles P_i/mg protein/hr) of PPLC-treated enzyme preparations. DOC-treated enzyme preparations were further treated with 50 U PPLC/mg protein for 60 min at 37°C. Points represent the mean ± SEM of 4 values.

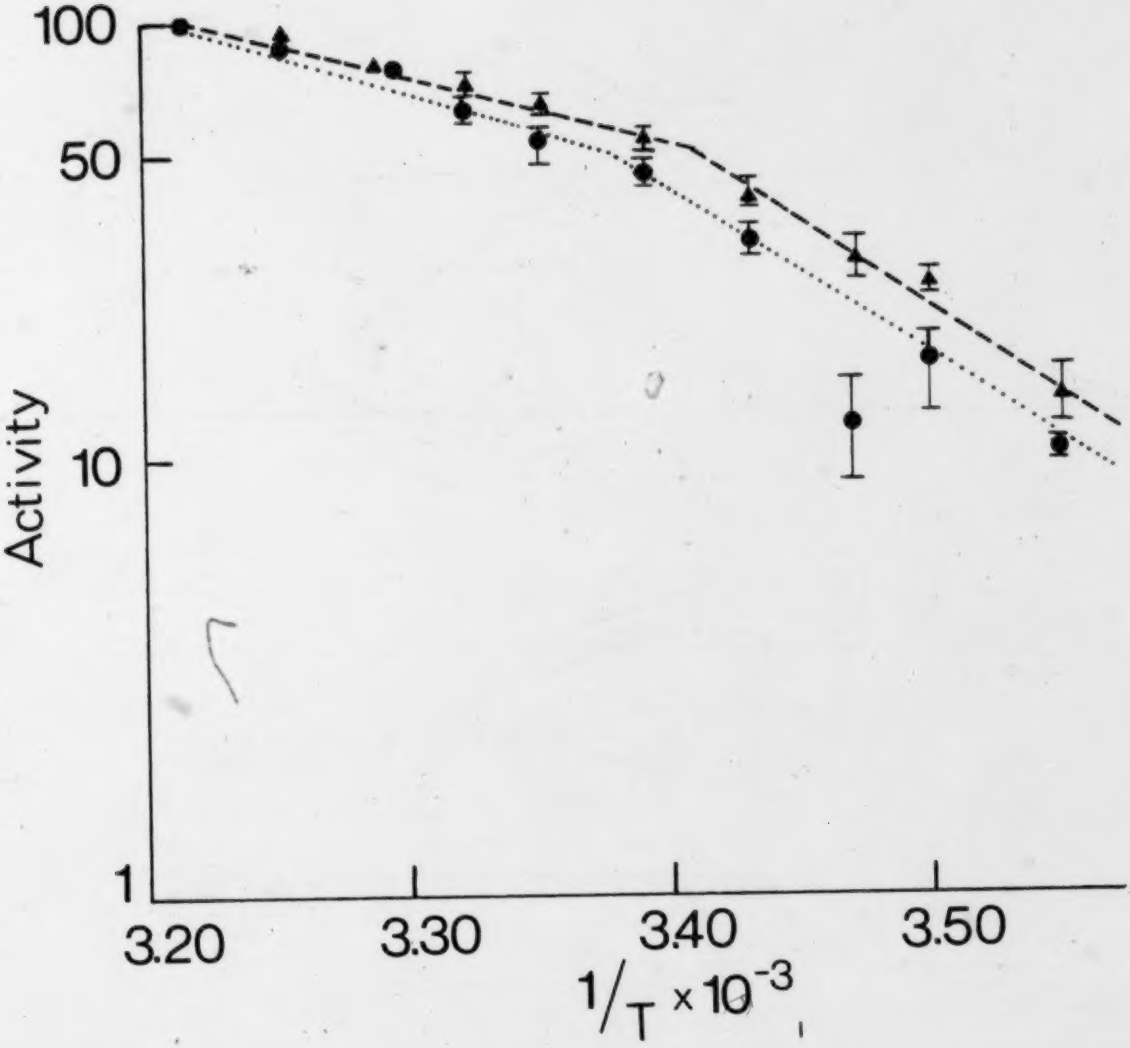

Fig. 21b Arrhenius plot of K$^+$-*p*-NPPase activity (▲) and O.I.-*p*-NPPase activity (●) of PPLC-treated enzyme preparations. Treatment conditions are described in the legend to Fig. 21a. Activities are expressed as umoles P_i/mg protein/hr. Points represent the mean ± SEM of 4 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

was linear (Fig. 22a), but the Arrhenius plots of K^+- and O.I.-*p*-NPPase activities were curvilinear (Fig. 22b). The curvilinear nature of this relationship is better presented by a curve of Activity vs Temperature (°C) (Fig. 22c). Again, modification of the lipid environment of the enzymes by PPLC treatment altered the temperature dependence of both enzyme activities in a different manner. The temperature dependence was different from control as well as from that seen after PPLA lipolysis.

The activation energies of the PPLC-treated preparations are presented in Table 12.

(c) (PPLA + PPLC) lipolysis

When a DOC-treated enzyme preparation was treated with both (PPLA + PPLC) as described in Methods (section 4 (c)),the temperature dependence of the (Na^+ + K^+)-ATPase was again altered to display a linear Arrhenius plot (Fig. 23a). The activation energy calculated from the slope of this plot was intermediate between the two values obtained from plots of the control untreated and DOC-treated preparations (Table 13). The activation energy was also similar to the value obtained from Arrhenius plots of the (Na^+ + K^+)-ATPase activity of a DOC-treated preparation further treated with either PPLA or PPLC, alone (Tables 10, 11 and 12).

The temperature dependence of K^+- and O.I.-*p*-NPPase did not change from that of control preparations. The mean Arrhenius plot of phosphatase activity was non-linear (Fig. 23b) and could be described as two straight lines intersecting at 20°C. The activation energies presented in Table 13 were similar to the values obtained from Arrhenius plots of control untreated and DOC-treated preparations as well as DOC-treated preparations which were further treated with either PPLA or PPLC alone (Tables 10, 11 and 12).

Therefore, treatment of a DOC-treated enzyme preparation with both lipases in combination altered the temperature dependence of both (Na^+ + K^+)-

ATPase activity as well as K$^+$-O.I.-*p*-NPPase activity in the same manner that treatment with either lipase alone had done.

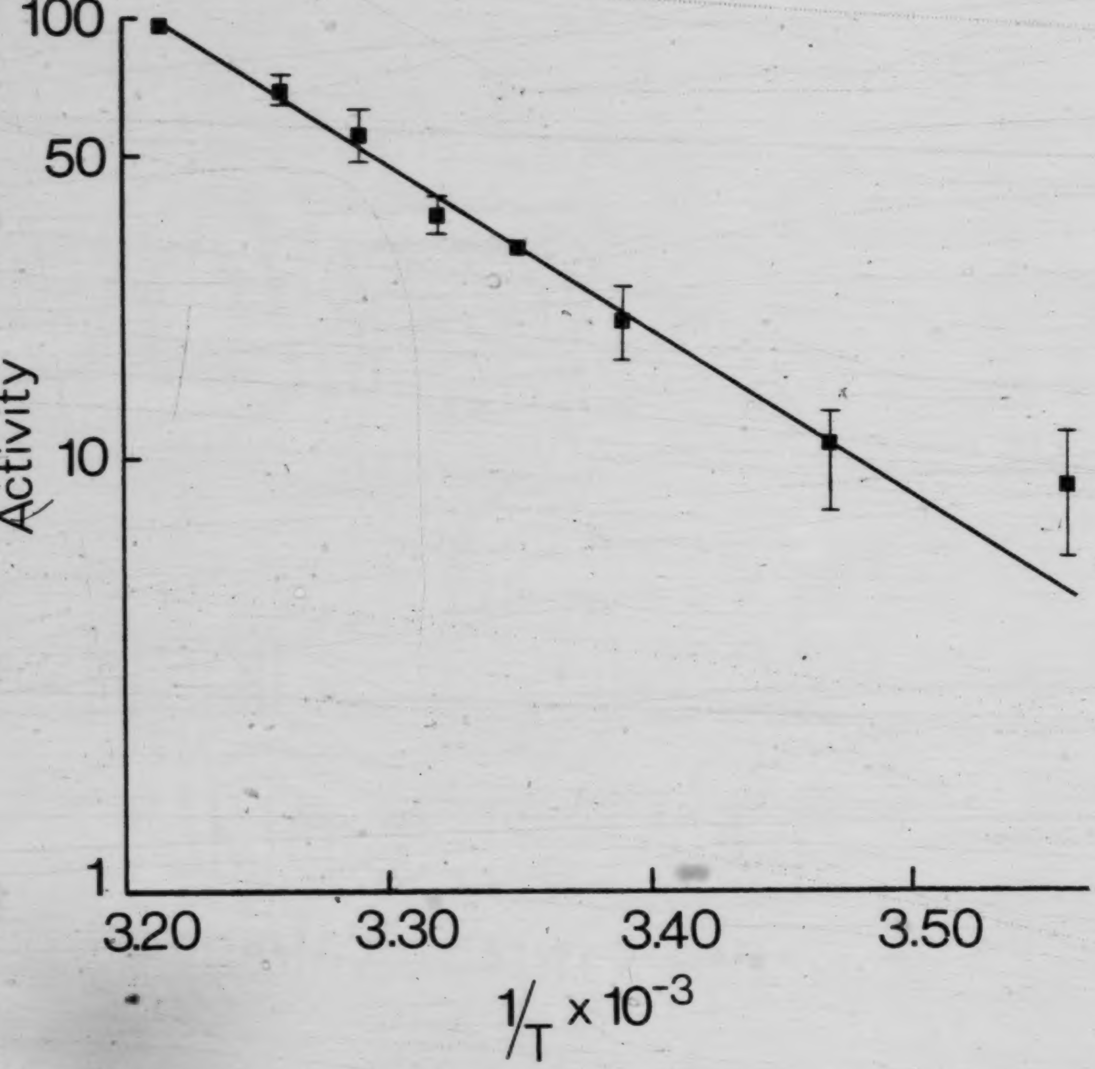

Fig. 22a Arrhenius plot of (Na^+ + K^+)-ATPase activity (umoles P_i/mg protein/hr) of PPLC-treated enzyme preparations. DOC-treated enzyme preparations were further treated with 50 U PPLC/mg protein for 60 min at 37°C in the presence of EDTA. Points represent the mean ± SEM of 4 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

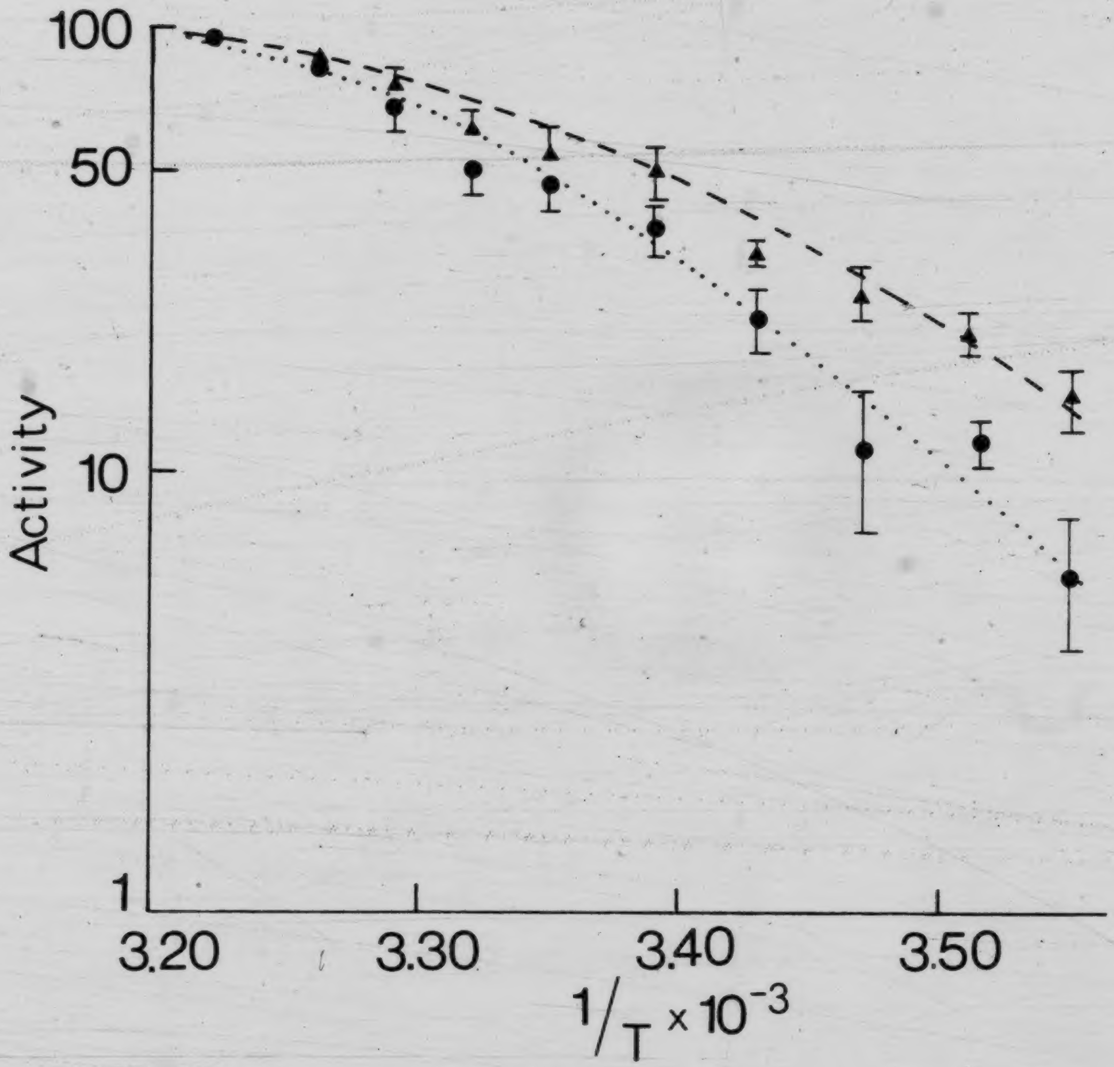

Fig. 22b Arrhenius plot of K^+-*p*-NPPase activity (▲) and O.I.-*p*-NPPase (●) activity of PPLC-treated enzyme preparations. Treatment conditions are described in the legend to Fig. 22a. Activities are expressed as umoles P_i/mg protein/hr. Points represent the mean ± SEM of 4 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

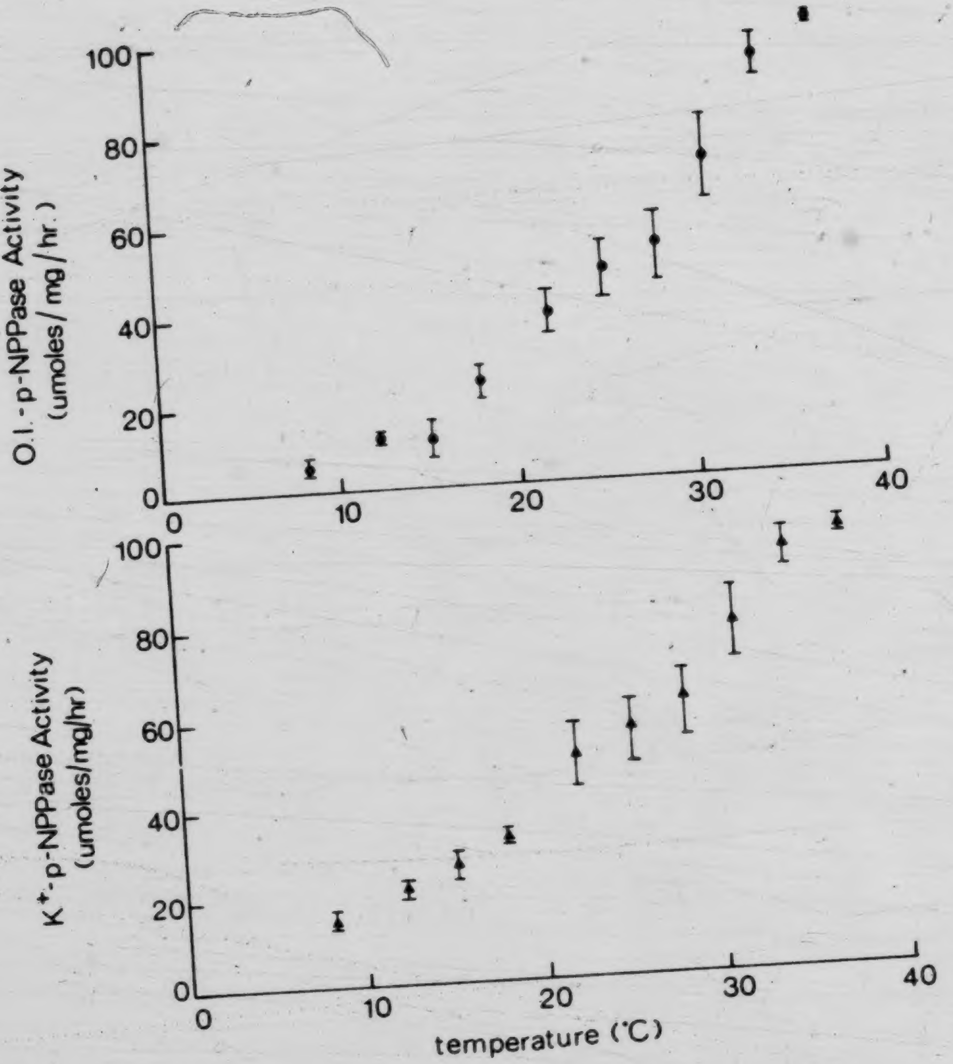

Fig. 22c Relationship between temperature and K^+-*p*-NPPase activity (▲) and O.I.-*p*-NPPase activity (●) of PPLC-treated enzyme preparations. Treatment conditions are described in the legend to Fig. 22a. Activities are expressed as umoles P_i/mg protein/hr. Points represent the mean ± SEM of 4 values.

TABLE 12

Temperature dependence of PPLC-treated enzyme preparations.

Treatment conditions (lipase concentration, incubation time, 37°C)	Enzyme activity	n	Ea_I*	Ea_{II}*	$Ea_I:Ea_{II}$	Tc*	Remarks
3-80 U PPLC/mg protein, untreated preparation, 60 min	$(Na^+ + K^+)$-ATPase	5	18.2	30.1	0.60	21	non-linear
	K^+-*p*-NPPase	9	----	----	----	--	non-linear**
	O.I.-*p*-NPPase	9	----	----	----	--	non-linear**
50 U PPLC/mg protein, DOC-treated preparation, 60 min	$(Na^+ + K^+)$-ATPase	4	22.9	----	----	--	linear
	K^+-*p*-NPPase	4	7.3	17.6	0.42	21	non-linear
	O.I.-*p*-NPPase	4	8.2	17.7	0.46	22	non-linear
50 U PPLC/mg protein, DOC-treated preparation in presence of EDTA, 60 min	$(Na^+ + K^+)$-ATPase	4	18.2	----	----	--	linear
	K^+-*p*-NPPase	4	----	----	----	--	non-linear***
	O.I.-*p*-NPPase	4	----	----	----	--	non-linear***

* Values for the activation energies above (Ea_I) and below (Ea_{II}) the transition temperature (Tc, °C) are expressed as kcal/mole.

** Activation energies were calculated from the slope of a line which could be drawn through the points between 34°C and 12°C: Ea (K^+-*p*-NPPase) = 10.4 kcal/mole; Ea (O.I.-*p*-NPPase) = 12.5 kcal/mole.

*** Meaningful activation energies could not be calculated from these curves.

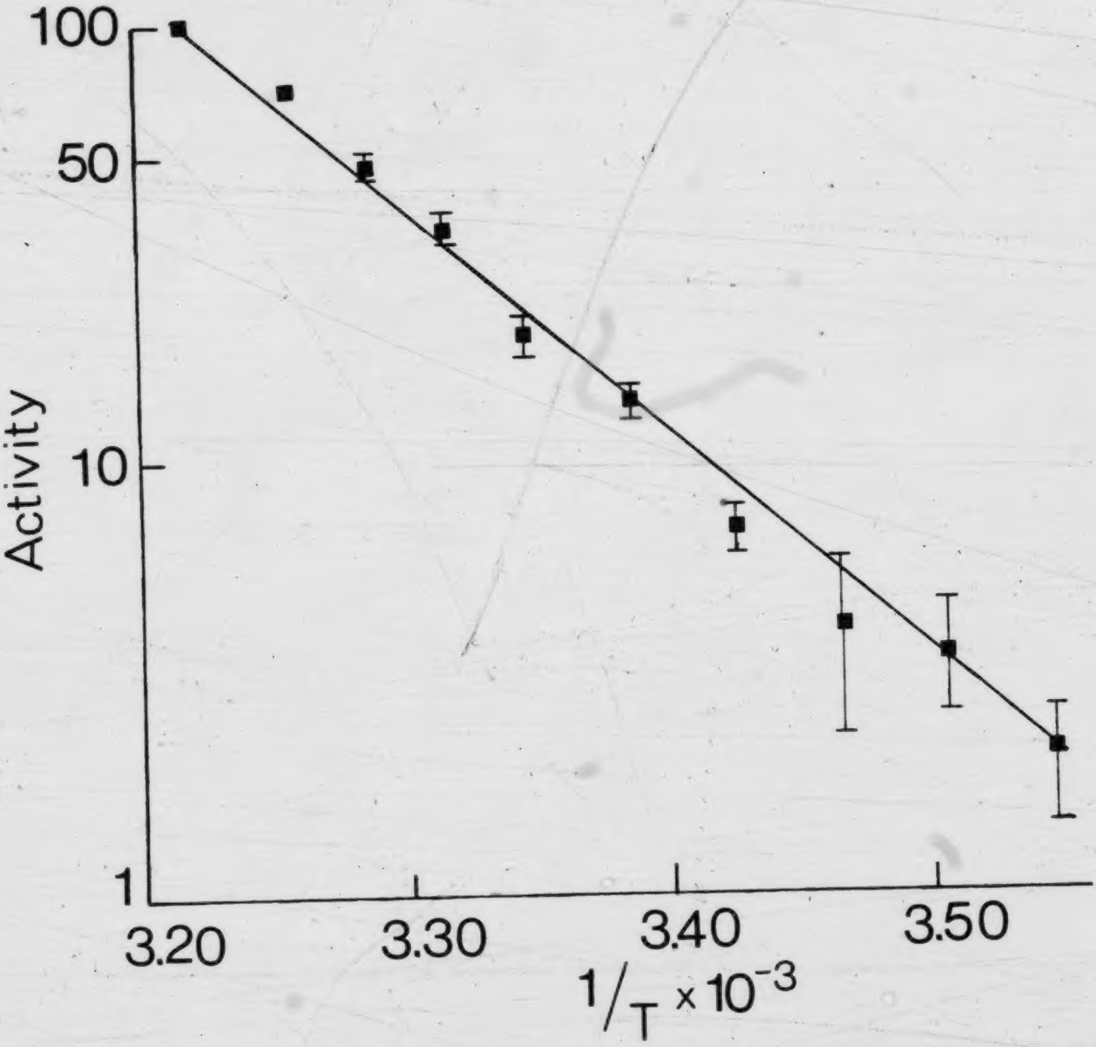

Fig. 23a Arrhenius plot of $(Na^+ + K^+)$-ATPase activity (μmoles P_i/mg protein/hr) of (PPLA + PPLC) treated enzyme preparations. DOC-treated preparations were treated with 5 U PPLA/mg protein for 11 min followed by 50 U PPLC/mg protein for 60 min at 37°C as described in the text. Points represent the mean ± SEM of 4 values. Where no error bars are shown, the SEM is small and lies within the dimensions of the point.

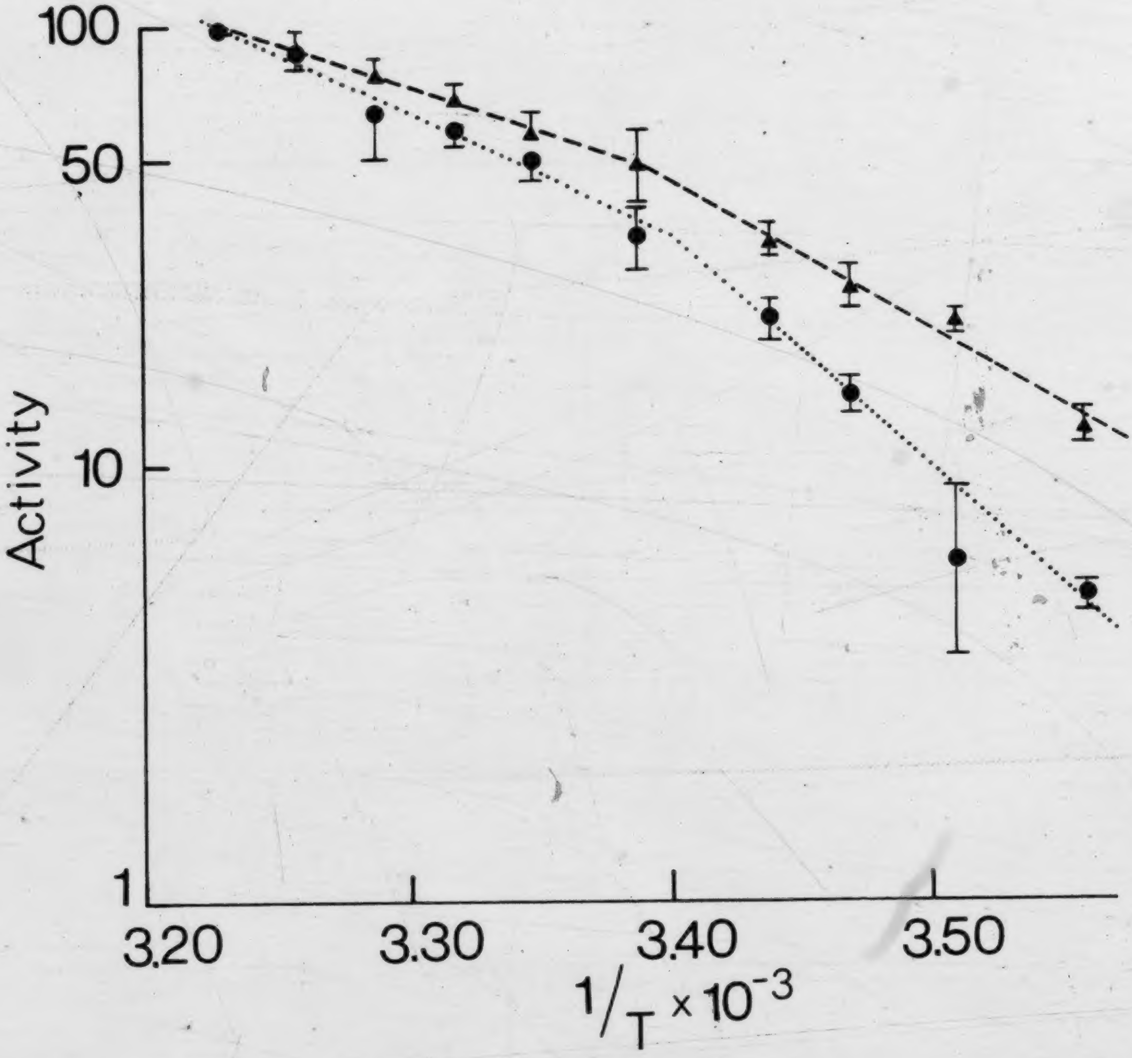

Fig. 23b Arrhenius plot of K^+-*p*-NPPase activity (▲) and O.I.-*p*-NPPase activity (●) of (PPLA + PPLC) treated enzyme preparations. Treatment conditions are described in the legend to Fig. 23a. Activities are expressed as umoles P_i/mg protein/hr. Points represent the mean ± SEM of 4 values.

TABLE 13

Temperature dependence of (PPLA + PPLC) treatment of DOC-treated enzyme preparations

Treatment conditions (lipase concentration, incubation time, 37°C)	Enzyme activity	n	Ea_I*	Ea_{II}*	Ea_I:Ea_{II}	Tc*	Remarks
5 U PPLA/mg protein, 11 min; 50 U PPLC/mg protein, 60 min	$(Na^+ + K^+)$-ATPase	4	25.7	----	----	--	linear
	K^+-*p*-NPPase	4	8.4	16.9	0.50	22	non-linear
	O.I.-*p*-NPPase	4	12.9	24	0.54	22	non-linear

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*Values for the activation energies above (Ea_I) and below (Ea_{II}) the transition temperature (Tc, °C) are expressed as kcal/mole.

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DISCUSSION

At the outset of the present studies, the lipid dependence of the ($Na^+ + K^+$)-ATPase reaction was already established (32, 33, 9, 68, 84, 100, 121, 138, 145, 146). The phosphatase reaction which is a partial reaction of the overall ($Na^+ + K^+$)-ATPase reaction sequence appeared to be lipid dependent as well, although a precise lipid requirement had not yet been elucidated (57, 143, 145, 147, 153, 156, 157, 160). Therefore, our present studies using phospholipase treatments of beef brain membrane preparations, were undertaken in an attempt to answer the following questions:

1. is there a requirement for a particular lipid(s) to modulate the phosphatase reaction? and
2. if so, what is the function of the lipid(s) in the phosphatase reaction?

We sought an answer to these questions by examining the effect of various procedures which modify membrane lipids on three parameters of the overall ($Na^+ + K^+$)-ATPase activity as well as of the partial phosphatase activity:

1. specific activity,
2. ouabain inhibition, and
3. temperature dependence.

The criteria for establishing these parameters was described at the beginning of the Results section. During the course of this work, it was found that virtually identical values were obtained for phosphatase activity whether it was measured as K^+-stimulated-*p*-NPPase activity or as ouabain-inhibitable-(O.I.)-*p*-NPPase activity. Therefore, for brevity and consistency, henceforth in this discussion, the partial reaction will be referred to as the K^+-O.I.-*p*-NPPase reaction, or simply as the phosphatase reaction.

Several investigators have treated ($Na^+ + K^+$)-ATPase containing membrane preparations with phospholipases, but have usually only examined the specific activity of the overall ($Na^+ + K^+$)-ATPase reaction, although some have examined the phosphatase reaction, as well (57, 72, 121, 147, 156, 157). Occasionally, the ouabain-inhibition of these reactions was also examined (59, 76, 147, 156). However, the temperature dependence of these reactions was examined in only very few studies (72, 146, 153) besides our own (30, 32, 33).

Kimelberg and Papahadjopoulos (72) examined the temperature dependence of an ammonium sulfate fraction of a ($Na^+ + K^+$)-ATPase preparation from rabbit kidney outer medulla after re-introducing various phospholipids into the system. They found that the inflection point of an Arrhenius plot of ($Na^+ + K^+$)-ATPase activity of a relipidated membrane preparation corresponded to the beginning or end of the transition range of the membrane system as determined by differential scanning calorimetry. Tanaka and Teruya (146) studied the effects of exogenous lipids on the temperature dependence of ($Na^+ + K^+$)-ATPase activity from bovine cerebral cortex and bullfrog kidneys which had been treated with DOC and NaI. They found that the appearance of the activity-temperature curve depended on the type of lipid in the system especially the nature of its fatty acyl chains, but not on the source of the enzyme. In a recent study which coincided with our own, Wheeler *et al.* (155) examined the temperature dependence of not only the ($Na^+ + K^+$)-ATPase activity, but also the K^+-O.I.-*p*-NPPase activity of microsomal fractions of rabbit kidney treated with NaI and Lubrol. They found that the inflection in the Arrhenius plot of their "K^+-dependent" phosphatase activity could be abolished by detergent treatment with Lubrol following extraction with NaI. However, the Arrhenius plot of "ouabain-sensitive"-phosphatase activity was still biphasic unless the ouabain concentration in the assay medium was increased ten-fold to 2.0 mM.

In our own work, temperature-activity relationships of (Na$^+$ + K$^+$)-ATPase as well as of K$^+$-O.I.-p-NPPase of PPLA- and PPLC-treated microsomal fractions of previously untreated and detergent treated beef brain were examined. At all temperatures with all treated and untreated preparations, K$^+$-O.I.-p-NPPase activity was determined by assaying in the presence of 2.0 mM ouabain which maximally inhibited K$^+$-p-NPPase activity. The appearance of the Arrhenius curves of K$^+$-O.I.-p-NPPase activity was not always similarly altered after various lipolytic treatments. The implications of these results will be discussed below.

Our results can be briefly summarized as follows: Phospholipase-treatment of untreated or DOC-treated beef brain microsomes lowered the specific activity of both (Na$^+$ + K$^+$)-ATPase as well as K$^+$-O.I.-p-NPPase, although not always in parallel, did not greatly alter the ouabain inhibition of either enzyme reaction, and altered the temperature dependence of both enzyme reactions in different ways depending on the lipolytic treatment.

The answer to the first question, "Is there a requirement for a particular lipid(s) to modulate the phosphatase reaction?" could not be obtained entirely by our methods. Lipid analyses would need to be performed on both the treated membrane preparation and on the products of lipolysis. After identifying the lipids remaining in the membrane and/or the reaction products of those lipids which were lyzed during lipolysis, a correlation could be made between the altered lipids and the changes in the values of the parameters examined. Thereby, the specific lipid requirement(s) of the overall (Na$^+$ + K$^+$)-ATPase reaction and the partial K$^+$-O.I.-p-NPPase reaction could be derived. Our results did, however, permit us to answer this question in rather general terms by differentiating between the requirements for lipids of the bulk membrane phase and the lipids which are more tightly bound.

However, our present studies do provide an answer to the second question. "What is the function of the lipids in the phosphatase reaction?" Their function was determined by examining changes in the three parameters of enzyme activity after lipolysis. A proposed model of lipid modulation of the ($Na^+ + K^+$)-ATPase reaction and of the K^+-O.I.-*p*-NPPase reaction was then constructed and is presented in the concluding section of this dissertation.

These results confirm the belief that lipids are involved in modulating the phosphatase reaction. Their method of doing so will be elaborated upon in the discussion which follows.

A. Preliminary Experiments

1. Controls: untreated and DOC-treated enzyme preparations.

In this work, it was necessary to consider both untreated as well as DOC-treated enzyme preparations as controls, since both preparations were used as substrates for subsequent phospholipase treatments.

As described in Table 6, DOC-treatment enhanced ($Na^+ + K^+$)-ATPase activity four-fold and K^+-O.I.-*p*-NPPase activity two-fold. This non-parallel increase in specific activities could suggest that if both activities were part of the same enzyme complex, then each activity had a different lipid requirement. More specifically, the phosphatase activity might not be as lipid dependent as the overall ($Na^+ + K^+$)-ATPase activity. Of course, the disproportionate increase in activities could also suggest that the overall ($Na^+ + K^+$)-ATPase and partial K^+-O.I.-*p*-NPPase reactions occur on two separate enzyme molecules which are not similarly affected by DOC-treatment. Mild detergent treatment (as we employed) would extract some extrinsic proteins of the membrane as well as solubilize the lipids of the bulk phase of the membrane matrix rather than those lipids which have been postulated to be more closely associated with certain membrane proteins in a type of boundary zone or annulus surrounding them (101, 161). Therefore, our results suggest that the lipids of the bulk membrane phase contribute more to modulating the overall ($Na^+ + K^+$)-ATPase reaction rather than the phosphatase reaction.

Another result of DOC-treatment was an enhanced ouabain-inhibitable fraction of both ($Na^+ + K^+$)-ATPase and K^+-O.I.-*p*-NPPase activities at 37°C. However, it should be noted that the ouabain-inhibitable portion of the K^+-activated-*p*-NPPase activity of untreated preparations was already 90-95% before detergent-treatment, and therefore, could not be enhanced much more by any treatment. The fact that the ouabain inhibition of both enzyme activities was enhanced by the same amount (5%) could suggest that the site of ouabain inhibition is the same for both enzyme activities. Besides suggesting that both enzyme activities occur on the same macromolecule, this result would also add to the evidence that the partial phosphatase activity is the specific locus of ouabain inhibition in the overall ($Na^+ + K^+$)-ATPase reaction sequence.

Because the percentage of either enzyme activity which could be inhibited by ouabain was not changed by a large amount, the site of ouabain inhibition was perhaps not dependent on the lipids extracted by DOC-treatment, i.e., the lipids of the bulk phase.

The temperature-activity relationship of both ($Na^+ + K^+$)-ATPase as well as K^+-O.I.-*p*-NPPase was not altered from that of untreated control preparations by DOC-treatment. Because the Arrhenius plots of both activities remained non-linear and produced essentially the same values for activation energies as did plots of the activities of untreated preparations, and because non-linearity has been ascribed to the change in physical state of the membrane lipids (32, 72, 146, 155), therefore, both reactions remained lipid-dependent after DOC-treatment. It could be suggested that the detergent DOC had not extracted those lipids which modulated the overall $(Na^+ + K^+)$-ATPase reaction and the partial K^+-O.I.-*p*-NPPase reaction. These results could also suggest that extraction of certain extrinsic proteins by DOC had not altered the packing or structure of the remaining membrane lipids sufficiently to alter their modulation of the enzyme reactions studied. Nevertheless, the similar lack of change in this parameter in both $(Na^+ + K^+)$-ATPase activity and in K^+-O.I.-*p*-NPPase activity again suggested that both activities could occur on the same enzyme macromolecule and could possess similar lipid dependence.

The value of Ea_{II}, the activation energy calculated from the slope of the Arrhenius plot below the inflection point, was twice as high for the $(Na^+ + K^+)$-ATPase activity as it was for the K^+-O.I.-*p*-NPPase activity in treated and untreated preparations. The higher value of Ea_{II} at low temperatures suggests that the energy requirement of the overall reaction is greater than that of the partial reaction when the lipids are in a more rigid state, i.e., the overall reaction is more dependent on the physical state of the membrane lipids than is the partial reaction. The fact that the phosphatase requires relatively less energy at the lower temperatures could give further support to the postulate that the phosphatase activity as a partial reaction of $(Na^+ + K^+)$-ATPase could occur on a portion of the same enzyme complex as the overall reaction, but would possess a different and probably less stringent lipid requirement than the overall reaction.

The similar range for the transition temperature (20-25°C) for both $(Na^+ + K^+)$-ATPase and K^+-O.I.-*p*-NPPase activities before and after detergent treatment would suggest that both activities were being modulated by similar lipids, or at least by those lipids whose transition temperatures overlapped. The modulating lipids could be a part of the molecular complex which contained both enzyme activities and could influence both activities similarly. The similar inflection point before and after detergent treatment could suggest that the modulating lipids were not those which were extracted by DOC. If DOC treatment did remove some modulating lipids, then our results suggest that suitable lipids remained to maintain the temperature-activity relationships of the enzyme as in the untreated preparation.

Our results differ from those of Tanaka and Mitsumata (144) who reported that DOC lowered the (Na^+ + K^+)-ATPase activity of bovine cerebral cortex. However, our results agree with those of many other investigators including Kimelberg and Papahadhopoulos (72) who found that DOC enhanced the (Na^+ + K^+)-ATPase activity of rabbit kidney outer medulla. A general review of the literature leaves the impression that DOC and other detergents are expected to remain within the membrane preparations and influence the values of various biochemical parameters which can be measured thus influencing the outcome of subsequent treatments. However, Kimelberg and Papahadjopoulos have found that dialysis effectively removed 90% of the DOC from their preparation. In our own laboratory, A.F. Almeida (unpublished results) found that our washing procedure by repeated centrifugation (Methods, section 3) leaves negligible amounts of DOC within the membrane. Wheeler *et al.* (155) seem to imply that residual Lubrol was left within their membrane preparation, but found that its presence was not responsible for the observed decrease in (Na^+ + K^+)-ATPase activity. Their results demonstrated a differential effect of detergent treatment on (Na^+ + K^+)-ATPase and K^+-O.I.-*p*-NPPase activities which was not peculiar to Lubrol, but occurred also with DOC (156). Detergent treatment lowered (Na^+ + K^+)-ATPase activity more drastically than phosphatase activity which could still be measured even when a second detergent treatment abolished the overall $(Na^+ + K^+)$-ATPase activity. After several repeated treatments, however, subsequent analyses revealed that all the lipids had not been extracted from the membrane preparation but neither $(Na^+ + K^+)$-ATPase nor K^+-O.I.-*p*-NPPase activities were detectible. Like our own observations, their studies led to the conclusion that the lipid dependence of the partial phosphatase reaction was different than that of the overall $(Na^+ + K^+)$-ATPase and was perhaps less stringent.

2. Lipolysis

When (PPLA + PPLC) were used together to treat a DOC-treated enzyme preparation, certain preliminary experiments were necessary to serve as controls for the double lipase treatment. These preliminary experiments presented interesting findings on their own besides serving as controls.

(PPLA + PPLC) double treatments were performed on DOC-treated enzyme preparations as described in Methods (section 4, c). Because the action of PPLA, which was added first to the incubation medium, was terminated by chelating Ca^{++} with EDTA, it was then necessary for PPLC to react with a DOC-treated enzyme preparation in the presence of excess EDTA and chelated CaEDTA. Because PPLC originally acted on an untreated enzyme preparation, it was now necessary to examine the effects of PPLC-treatment of a DOC-treated enzyme preparation in the presence and absence of EDTA.

When PPLC-lipolysis was carried out on a DOC-treated preparation, $(Na^+ + K^+)$-ATPase as well as K^+-O.I.-*p*-NPPase activities were lowered further than when an untreated preparation was the substrate (Table 7-B). These values approached the range which was obtained when PPLA was used to treat a DOC-treated preparation (Table 7-A). This result could imply that pre-treatment of an enzyme preparation with the detergent DOC enhanced the effectiveness of lipase action. The extraction of certain lipids and extrinsic proteins from the membrane matrix by detergent action could make the remaining phospholipids, especially those associated with the modulation of enzyme activity, more accessible to lipase action.

The second PPLC lipolysis which served as a control for a (PPLA + PPLC) double treatment was performed on a DOC-treated enzyme preparation in the presence of EDTA in the same concentration as would have been necessary to inactivate PPLA had it been present. Here, again, both (Na^+ + K^+)-ATPase and K^+-O.I.-*p*-NPPase activities were lowered even further (Table 7-B). The chelation of Ca^{++} and other divalent metal ions by EDTA did not reduce PPLC activity, in fact, it seemed to enhance it. Therefore, Ca^{++} appears to be unnecessary for the action of PPLC from *B. cereus*. It has been postulated that Ca^{++} ions mediate electrostatic interactions between the membrane lipids by binding the polar head groups together forming a type of lattice (78, 79, 108, 167). In addition, Ca^{++} ions also mediate electrostatic interactions between lipids and extrinsic proteins (167). Therefore, it is feasible that removal of Ca^{++} ions could expose the bond between the phosphate head group and glycerol carbon making it more available for lysis by PPLC. This proposal would agree with one made by Kimelberg (71) that a metal ion bound to a phospholipid molecule, tended to enhance (Na^+ + K^+)-ATPase activity and the subsequent removal of the metal ion by chelation by EDTA would then lower the (Na^+ + K^+)-ATPase activity.

Taguchi and Ikezawa (141, 142) similarly found that the action of PPLC from various bacterial sources was stimulated in the presence of DOC. However, in contradiction to our own results, they found that divalent metal ions were necessary to enhance PPLC activity in the presence of DOC. In particular, they reported that Ca^{++} stimulated PPLC activity from not only *B. cereus*, but also *Cl. perfringens* and *Cl. novyi*, Type A.

The results of Low *et al.* (81) however, agreed with ours. They found that Ca^{++} was not required for the activity of PPLC from rat lymphocytes and indicated that PPLC's from different sources hydrolyzed different phospholipid substrates.

Martonosi *et al.* (87) investigated the effects of treating a sarcoplasmic reticulum membrane preparation from rabbit or rat muscle with DOC following PPLC treatment, i.e., in reverse order to our treatments. They found that this treatment enhanced (Ca^{++} + Mg^{++})-ATPase activity but it did not restore Ca^{++} transport function.

3. Summary of preliminary experiments

The preliminary experiments were meant to be a basis of comparison for subsequent phospholipase treatments. However, on their own, the results from this group of experiments suggest an answer to the question of the function of the lipids in the phosphatase reaction. The results suggest that lipids which can be extracted by mild detergent treatment do not greatly alter the parameters of enzyme activity except for enhancing the specific activity significantly. Also, detergent pretreatment appears to enhance the effectiveness of lipase action.

B. Specific Activity

1. Lipolysis

The parallel reduction of both (Na^+ + K^+)-ATPase as well as K^+-O.I-*p*-NPPase activities by PPLA, PPLC and (PPLA + PPLC) treatments supported the belief that these two activities could be part of the same enzyme complex[1]. PPLA-treatment of a DOC-treated enzyme preparation lowered both activities to the same level of 25% of control (Table 7-A) suggesting that the phospholipids which are substrates for PPLA could be evenly distributed between the active sites of these two activities. PPLC-treatment, however, lowered the activities by a greater amount if a DOC-treated enzyme preparation was used as a substrate, rather than an untreated preparation, especially if EDTA was present during the incubation (Table 7-B). The implications of this result have been discussed above. Although both activities were reduced in parallel, the $(Na^+ + K^+)$-ATPase activity was lowered to a greater extent than was K^+-O.I-*p*-NPPase activity suggesting that the phospholipids which are substrates for PPLC and which modulate these enzyme activities are unevenly distributed between the two active sites. Because the site of Na^+ and ATP binding (initiating the kinase reaction) is on the inside of the membrane, while the site of K^+ binding (initiating the phosphatase reaction) is on the outside of the membrane, these reactions can be described as being asymmetrically distributed across the membrane (although both could be described as being "transmembrane" as in the Introduction, section B 2). Therefore, if modulating phospholipids are unevenly distributed between the two active sites, there could also be an asymmetric distribution of phospholipids across the membrane bilayer. The results of PPLC-treatments could also suggest that the K^+-O.I-*p*-NPPase activity is not as lipid dependent as the overall $(Na^+ + K^+)$-ATPase activity or its lipid requirement is not as stringent. Tanaka (143) has suggested that more varied lipids can modulate the phosphatase reaction. Of course, the above results could also suggest that these two enzyme activities occur on different molecules which have different phospholipid requirements.

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[1] (The increase in activity which was observed after treatment with low concentrations of PPLA or PPLC for short periods of time was attributed to the exposure of more active sites after removal of a comparatively small amount of membrane phospholipids by the lipases. A more detailed explanation has been presented by Charnock *et al.* (32) in describing results previously obtained in this laboratory).

When (PPLA + PPLC) were both used to treat a DOC-treated enzyme preparation, $(Na^+ + K^+)$-ATPase activity was reduced to 20% of control values which was the level attained by PPLA treatment alone, and K^+-O.I.-*p*-NPPase activity was reduced to 40% of control, a level which was attained after PPLC treatment alone. Again, the asymmetric distribution of the phospholipids between the two active sites across the membrane bilayer is suggested. It is difficult to find a suitable explanation for the lowering of both activities to these levels. Since PPLA was added to the incubation medium first, both ($Na^+ + K^+$)-ATPase as well as K^+-O.I.-*p*-NPPase activities could be expected to be lowered to 25% of control. The subsequent addition of PPLC could be expected to lower ($Na^+ + K^+$)-ATPase activity further to about 10% of control as in the preliminary PPLC-treatment of a DOC-treated preparation in the presence of EDTA (Table 7-B). However, under these same conditions, PPLC lowered K^+-O.I.-*p*-NPPase activity to 30% of control. Therefore, interpretation of the results after (PPLA + PPLC) treatment remains difficult.

Azhar *et al.* (16) have postulated a novel mechanism for the observed restoration of gonadotropin receptor binding activity and of lipid-protein interactions after PPLC-treatment of plasma membranes of bovine corpus luteum. They suggested that removal of polar head groups permitted an elongation of the apolar moieties [diglycerides] remaining in the membrane and also an aggregation of the diglycerides into lipid droplets. Verkleij *et al.* (152) supported this postulate after they saw such droplets on the freeze fracture surface of erythrocyte membranes after PPLC-treatment. (In contrast, after PPLA-treatment, irregular particulate clusters were seen.) A similar type of elongation and increased mobility of the few remaining phospholipids in our beef brain membrane preparation after combined (PPLA + PPLC) treatment could account for the partial restoration of K^+-O.I.-*p*-NPPase activity after treatment with PPLC following treatment with PPLA.

No matter which lipase treatment was used, or for whatever duration of incubation, a certain residual amount of both ($Na^+ + K^+$)-ATPase and of K^+-O.I.-*p*-NPPase activities remained. The residual activities could be attributed to a certain group of tightly bound lipids (perhaps forming an annulus) which surrounded the active sites and which were sufficient to modulate their activities. These lipids could have remained intact after lipolytic treatments because they may not have been substrates for PPLA or for PPLC action by virtue of their structure or their location within the membrane matrix very near the enzyme. An equally plausible explanation could be that the residual activity was independent of a lipid environment.

Alternatively, the products of lipolysis may have inhibited further lipolysis, and the complete abolition of ($Na^+ + K^+$)-ATPase and K^+-O.I.-*p*-NPPase activities would have been prevented. This latter possibility is rather unlikely since the presence of BSA in the incubation medium during PPLA lipolysis should have satisfactorily removed the products of PPLA lipolysis, the free fatty acids and the lysophospholipids (16, 76, 80, 121). The diglycerides which remained in the membrane after PPLC had removed the polar head groups are not thought to be inhibitors of enzyme activities (87, 147). However, we chose to investigate the possibility of product inhibition by performing a double-treatment with PPLA, but found that both ($Na^+ + K^+$)-ATPase and K^+-O.I.- -NPPase activities were not lowered beyond that amount which could be ascribed to the mechanical procedures of the lipase treatment. This result contrasts with that of Taniguchi and Tonomura (147) who found that their centrifuging procedures were not responsible for lowering enzyme activities.

Another method of testing for product inhibition would have been to add exogenous fatty acids or lyso-phospholipids to the enzyme preparation and then to measure ($Na^+ + K^+$)-ATPase and K^+-O.I.-*p*-NPPase activities. However, the value of this experiment could be dubious since Lin (80) has found that endogenously produced lysophospholipids (as well as phosphatidic acid) could have greater inhibitory effects at lower concentrations than exogenously added lysophopholipids would have. Also, Martonosi *et al.* (87) found that exogenously added lysophosphatidylcholine could restore [not inhibit] the ATPase activity as well as the Ca^{++} transporting activity of (Ca^{++} + Mg^{++})-ATPase of the sarcoplasmic reticulum of rat or rabbit skeletal muscle.

A residual amount of (Na^+ + K^+)-ATPase and K^+-0.1M-*p*-NPPase activities remained in our preparation even after prolonged incubation. Maximum reduction in activities was achieved after about 8 min lipolysis with PPLA at 37°C (Fig. 14) and after about 20 min at 20°C (Fig. 15); and after about 30 min lipolysis with PPLC at 37°C (Fig. 16a). Our results agree with those of Nilsson *et al.* (92) who similarly found that PPLA maximally inactivated (Na^+ + K^+)-ATPase activity of subcellular membranes of rat liver within the first 10 min of incubation even at 4°C. He found that phosphatidylserine and phosphatidylethanolamine were almost completely hydrolyzed and concluded that the other phospholipids were not available for lipolysis. Verkleij *et al.* (152) also found that prolonged incubation with PPLA or with PPLC failed to hydrolyze more phospholipids of the red blood cells.

Roelofsen and van Deenen (121) similarly found that treating red blood cell ghosts with phosphatidylserine decarboxylase left a residual 13% (Na^+ + K^+)-ATPase activity in the preparation which could only be abolished by treatment with anhydrous ether followed by another treatment with phosphatidylserine decarboxylase. When Isern de Caldenty and Wheeler (66) treated rabbit kidney with phosphatidylserine decarboxylase, they found that 17% of the membrane phosphatidylserine was not converted to phosphatidylethanolamine and decided that this residual 17% must be bound to the (Na^+ + K^+)-ATPase in a specific electrostatic manner which made it resistant to phosphatidylserine decarboxylase action. They further stated that the rest of the phospholipids must be bound in a non-specific hydrophobic manner.

Taniguchi and Tonomura (147) also found that PPLA and PPLC treatments lowered $(Na^+ + K^+)$-ATPase activity to 25% and 40% of control which they did not attempt to lower further. In contrast, Yorio *et al.* (162) have reported that PPLC-treatment of frog skin stimulated active Na^+ transport probably by lysing the phosphatidylcholine at the site of Na^+ influx. [$(Na^+ + K^+)$-ATPase activity was not measured but it could have been lowered or abolished while Na^+ influx was increased across a membrane disrupted by lipase treatments.]

Therefore the above experiments seem to indicate that there is a certain group of lipids associated with the $(Na^+ + K^+)$-ATPase and K^+-O.I.-*p*-NPPase active sites which is relatively resistant to lipase action under various conditions and which seems to be responsible for maintaining a certain residual amount of both enzyme activities after lipase treatments. It could be conceived that these lipids are "protecting" the enzyme active sites. Nilsson *et al.* (92) investigated the reverse situation, i.e., the possibility that the membrane proteins were protecting the phospholipids from further lipolysis by PPLA. However, after treating their rat liver subcellular membranes with proteases before adding PPLA, greater lipolysis did not occur. Therefore, according to these results, the fact that a residual amount of phospholipids remained in the membrane cannot be attributed to their close association with the proteins, but it is still possible that the residual phospholipids may not have been substrates for PPLA action.

We also investigated the possibility that ATP in the incubation medium might prevent the loss of $(Na^+ + K^+)$-ATPase and K^+-O.I.-*p*-NPPase activities during lipolysis. However, when it was found that both activities were lowered by the same amount with or without ATP in the incubation medium, we proposed that the ATP binding site was either not lipid dependent, or that the lipids at the binding site were not substrates for PPLA or PPLC lipolysis. However, Wheeler *et al.* (157) found that ATP binding to the enzyme to initiate the Na^+-dependent phosphorylation step was indeed lipid dependent. Therefore, our results only demonstrated that whether or not the lipids at the ATP binding site were substrates for PPLA or for PPLC lipolysis, they were then equally affected by the presence or absence of ATP in the incubation medium.

2. Summary of effects of lipolysis on specific activity

Examination of the specific activity of (Na^+ + K^+)-ATPase and K^+-O.I.-*p*-NPPase activities after lipase treatments suggests that the phospholipids do modulate these activities, and that the K^+-O.I.-*p*-NPPase activity is probably not as lipid dependent as the overall (Na^+ + K^+)-ATPase activity. We propose that the phospholipids are asymmetrically distributed across the membrane about the respective enzyme active sites, and that they modulate enzyme activities by aiding in the binding of substrates and other ligands to initiate the various steps of the reaction sequence. However, our results do not permit us to infer which specific lipids are involved in modulating enzyme specific activities. Such an asymmetric distribution of lipids could accomodate the postulate that both enzyme activities are part of the same macromolecule without invoking the existence of two separate enzyme molecules.

C. Ouabain Inhibition

The pharmacological interest in (Na^+ + K^+)-ATPase is due to its role as the putative cardiac glycoside receptor mediating the positive inotropic action of these drugs. The electrophysiological events which follow the inhibition of (Na^+ + K^+)-ATPase by cardiac glycosides have been reviewed by Lee and Klaus (77). Recent work by Zavecz and Dutta (168) with the synthetic cardiac glycoside actodigin has confirmed the relationship between the positive inotropic effects in the heart, and the inhibition of Na^+ and K^+ transport, and the inhibition of $(Na^+ + K^+)$-ATPase activity.

Our particular interest in this mechanism was to determine if the lipids of the membrane modulated the binding of ouabain to the $(Na^+ + K^+)$-ATPase, to effect its inhibition or the inhibition of the partial phosphatase reaction.

The binding of ouabain to $(Na^+ + K^+)$-ATPase has been extensively investigated in our own laboratory (35). After examination of the rate of binding as well as the equilibrium level of binding in various lipid depleted preparations, it appeared as if two ouabain binding sites existed, one of which was more lipid dependent than the other. Hansen (60) also proposed the existence of non-uniform ouabain binding sites after examining the $(Na^+ + K^+)$-ATPase of ox brain membranes by manipulating concentrations of Mg^{++} and P_i as well as other ligands. These latter sites could be made homogeneous with respect to ouabain by the addition of K^+, which competes for the same binding site as ouabain.

With all our delipidated preparations, preliminary experiments were performed to ensure that the concentration of ouabain in the assay media was sufficient to maximally inhibit both $(Na^+ + K^+)$-ATPase and K^+-O.I.-*p*-NPPase activities. It was found that ouabain virtually completely inhibited the phosphatase reaction of all the delipidated as well as untreated preparations. This result strengthened the argument that the phosphatase reaction was the specific site of ouabain inhibition of the overall $(Na^+ + K^+)$-ATPase reaction sequence (Introduction, section A), although it did not exclude the possibility that ouabain could inhibit one of the other steps in the reaction as well. This proposal could accomodate the existence of two ouabain binding sites, one of which was associated with the phosphatase reaction, and the other which could be associated with one of the other steps or could be relatively non-specific.

The fact that maximum inhibition of the partial phosphatase reaction required five times the concentration of ouabain than the overall $(Na^+ + K^+)$-

ATPase reaction still does not have a ready explanation. It is possible that some type of allosteric effect is operating when artificial substrates are used to measure phosphatase activity. The inhibitor binding at one site would be influencing the phosphatase reaction at its specific site of action and this influence would be different depending on whether the substrate of the phosphatase reaction were the natural one, $E_2 \sim P$, or an artificial one, such as *p*-NPP. When the dephosphorylation of the natural substrate $E_2 \sim P$ was being inhibited, 0.4 mM ouabain was sufficient for maximum inhibition, but 2.0 mM ouabain was necessary when *p*-NPP was the substrate. Ouabain binding and inhibition were proposed to be optimal after the enzyme had undergone the conformational change from $E_1 \sim P$ to $E_2 \sim P$ (59, 134), (although a lesser amount of ouabain binding to an E_1 form could be demonstrated under different conditions (134)). The $E_2 \sim P$ intermediate is highly unstable and has not been isolated (86), therefore, an examination of its breakdown cannot be carried out directly but requires the use of artificial substrates which invoke the phosphatase activity of the overall sequence. Since it appears that artificial substrates do not first phosphorylate the $(Na^+ + K^+)$-ATPase as the terminal phosphate is lyzed (68), perhaps the absence of a phosphorylated enzyme form is responsible for the allosteric effects on the phosphatase activity which in turn necessitate a higher concentration of ouabain to inhibit phosphatase activity when artificial substrates are used. A more precise explanation is not possible from our results.

However, the difference in concentration of ouabain required to maximally inhibit these two related enzyme activities could be explained by proposing that they resulted from two separate enzyme molecules with two different "sensitivities" to ouabain and are not part of the same enzyme macromolecule. Our results do not permit us to exclude either proposition.

1. Temperature dependence of ouabain inhibition

Previous results from our laboratory have demonstrated that the percentage of the overall ($Na^+ + K^+$)-ATPase activity of our preparations which could be inhibited by a constant concentration of ouabain increased with temperature (30, 33, 35). The present investigation duplicated these results with K^+-O.I.-*p*-NPPase as well as repeated them for ($Na^+ + K^+$)-ATPase (Tables 8-A, B, C, D). Above 20°C, the transition temperature of the membrane lipids (30, 32, 33), the percent ouabain inhibition of the phosphatase activity increased abruptly to be essentially 100%. The lower amount of ouabain-inhibitable phosphatase activity below 20°C was attributed to insufficient ouabain having bound to the enzyme due to a lower rate of binding at lower temperatures. Therefore, within the duration of the experiment, complete binding was not attained and maximum inhibition was not observed. This explanation assumes that the affinity of the binding sites was still the same at all temperatures within the range measured. To test this assumption, an aliquot of the enzyme preparation was preincubated with ouabain at 37°C to effect maximum binding before phosphatase activity was assayed at 4°, 8°, and at 10°C. When it was found that essentially 100% of the phosphatase activity was inhibited at the lower temperatures after this preincubation procedure (Table 9), it appeared as if the affinity of the ouabain binding site(s) was equal at all temperatures in the range examined, but the rates of association (and presumably dissociation) of ouabain were lower at the lower temperatures. At 37°C, maximum ouabain binding was known to occur within 25 sec (35). Because the rate of dissociation of ouabain from its binding site(s) is expected to be lower at lower temperatures, ouabain could reasonably be expected to remain bound to the enzyme - at least for the duration of the assay - as the preparation was cooled. Under these conditions, phosphatase activity might be expected to be completely inhibited at the lower temperatures, which is what occurred in our experiments.

The same type of situation as described in the above paragraph would be expected to occur in the ouabain-inhibitable-(Na^+ + K^+)-ATPase activity. However, when an enzyme preparation was preincubated with ouabain at 37°C to effect maximum binding and then assayed for ouabain-inhibitable (Na^+ + K^+)-ATPase activity at temperatures below 20°C, very little change in the amount of ouabain inhibition was noted from the control situation (35 and L.P. Simonson, unpublished results). Comparing this result to the inhibition of the phosphatase activity, it appears as if some ouabain is dissociating from the enzyme during the cooling process. This would be an unusual mechanism since the rate of dissociation is presumably slower at low temperatures. Alternatively, the affinity of the binding sites could be less at the lower temperatures. If the lipids are involved in ouabain binding, then it could be possible that they assist in ouabain binding at higher temperatures when they are in a more fluid liquid crystalline state, while they could hinder ouabain binding to its site when they are in a more rigid state at lower temperatures. In this manner, the state of the membrane lipids as dictated by the temperature could affect the affinity of the ouabain binding site of the overall (Na^+ + K^+)-ATPase reaction. Because the ouabain inhibition of the phosphatase reaction is not similarly affected, more evidence is presented to support the proposal that the partial K^+-O.I.-*p*-NPPase activity is not as lipid dependent as the overall (Na^+ + K^+)-ATPase reaction and is possibly modulated by a different group of lipids, and perhaps a more varied composition of lipids, than the (Na^+ + K^+)-ATPase activity. This proposal would especially apply to their respective ouabain binding sites. These results could also strengthen the evidence for two ouabain binding sites with different lipid requirements. That the extent of ouabain-inhibition of phosphatase activity could be shown to be 100% at all temperatures examined, supports the proposition that it is the specific locus of ouabain inhibition in the $(Na^+ + K^+)$-ATPase reaction sequence.

2. Effects of lipid depletion on ouabain inhibition

The lack of significant change in the amount of ouabain inhibition of $(Na^+ + K^+)$-ATPase or of the phosphatase reaction measured at 37°C after lipid depletion by the detergent DOC or by phospholipases suggested that this parameter was not particularly lipid dependent. The temperature dependence of ouabain inhibition was not greatly altered after lipid depletion except in the case of the PPLC-treated preparations. An examination of the amount of ouabain bound at equilibrium and the rate of binding to the enzyme after the various lipid treatments would have been necessary to accurately compare this parameter. Such examinations were not performed at this time except with a PPLC-treated preparation. Previous results of such an analysis had shown that DOC enhanced the rate and equilibrium level of ouabain-binding to $(Na^+ + K^+)$-ATPase and PPLA-treatments slightly reduced these values (35).

When an ouabain binding study was carried out by L.P. Simonson on a PPLC-treated enzyme preparation, it was found that both the equilibrium level of ouabain bound as well as the rate of binding were enhanced. Ouabain inhibition of the phosphatase reaction had been demonstrated to be virtually complete at all temperatures in all treated and untreated preparations (as discussed above). However, after PPLC-treatment the amount of ouabain inhibition of both $(Na^+ + K^+)$-ATPase and of the phosphatase appeared to be enhanced below 20°C even when the K^+-O.I.-*p*-NPPase activity was assayed without preincubating the preparation with ouabain (Tables 8-A, B, C, D). As the phosphatase assay of a PPLC-treated enzyme preparation was followed on the Gilford-2400 recording spectrophotometer, it was noted that the ouabain-insensitive *p*-NPPase activity achieved a steady-state level in a very short time, even at lower temperatures, (Fig. 18). The time to arrive at a steady-state was regarded as the time required for ouabain to bind. Therefore, this observation demonstrated that ouabain had bound to the enzyme within a very short time period, e.g., within 5 min. (Normally, ouabain binding to an untreated enzyme preparation is complete in 25 sec at 37°C, but it is not complete by 60 min at 8°C.)

A PPLC-treated enzyme preparation demonstrated a higher equilibrium level of ouabain binding, a faster rate of [^{3}H]-ouabain binding, a faster arrival at a steady-state level of ouabain-insensitive-phosphatase activity as observed on the Gilford traces, as well as an increased fraction of ouabain inhibitable enzyme activities below the transition temperature. These results all seemed to suggest that lipolysis with PPLC had removed some sort of impediment to ouabain binding. PPLC cleaves the bond between the polar head group and the carbon-1 of the phospholipid molecule, thereby removing a polar head group and leaving a diglyceride within the membrane matrix. Therefore, PPLC lipolysis would result in an altered charge distribution on the membrane surface. If the polar head groups had impeded ouabain binding, then these results would support the importance of the hydrophobic interactions of ouabain with the membrane constituents especially fatty acyl groups of a phospholipid molecule. Our results agreed with those of Martonosi *et al.* (87) who found that the same fraction of ouabain-inhibitable-ATPase activity remained after PPLC treatment as in the untreated enzyme preparation, but in our case, this fraction bound ouabain at a faster rate.

After double lipolysis with PPLA, the amount of ouabain-inhibitable-($Na^+ + K^+$)-ATPase at 37°C was still lowered to only 10-20% of control (see Results, section B, 3-c). Since the value of ouabain-inhibitable-($Na^+ + K^+$)-ATPase activity is considered as maximal at 37°C, this result would imply that some ($Na^+ + K^+$)-ATPase was inactivated by the double treatment, either by direct denaturation of the enzyme during the lipolytic procedure, or by the lack of sufficient lipid for activation. That the amount of ouabain-inhibition of the phosphatase activity was again unaltered after double lipolysis supports the evidence that there could be two ouabain binding sites on these preparations one of which is associated with the phosphatase reaction and which is not as sensitive to lipid depletion, and the other which is more sensitive to lipid depletion. If the first site is lipid-dependent, then it is probably modulated by a group of lipids which are tightly bound in the vicinity of the ouabain binding site and which are not affected by the various lipid treatments.

Although we did not find a significant difference between the fraction of ouabain inhibition at 37°C of a preparation treated with PPLC in the presence or absence of EDTA, Zachowski *et al.* (167) found that EDTA treatment alone removed some resistance to ouabain binding. By chelating the divalent ions of the membrane, EDTA would disrupt interactions between polar head groups of the phospholipid molecules as well as lipid-extrinsic protein interactions, thereby altering the charge distribution on the membrane. Therefore, the importance of hydrophobic binding of ouabain to fatty acyl residues would again be emphasized.

The absence of any significant change in the ouabain inhibition of either ($Na^+ + K^+$)-ATPase or of K^+-O.I.-*p*-NPPase could suggest that ouabain binding and inhibition are not lipid-dependent reactions. However, previous ouabain binding studies (35) and the present studies of the temperature dependence of ouabain inhibition do suggest that ouabain binding is indeed a lipid dependent function. Very recently Goodman and Walker (59) proposed a more precise function for the membrane phospholipids by stating that they are necessary to modulate the enzyme into a certain configuration which is more receptive to ouabain binding [most likely the $E_2 \sim P$ configuration]. Although our results may indicate that there is a requirement for phospholipids, they cannot specify their function more precisely than indicating that they are required to aid ouabain binding at the site on the enzyme; presumably this also could be the K^+ binding site.

3. Summary of ouabain inhibition

Our present results seem to suggest that ouabain inhibition of the overall (Na^+ + K^+)-ATPase reaction is more temperature dependent (and hence lipid dependent) than the ouabain inhibition of the partial K^+-O.I.-*p*-NPPase reaction, especially below the transition temperature of the treated and untreated membrane preparations. These results could support the proposition that there are two ouabain binding sites, one of which is associated with phosphatase activity and is modulated by a group of tightly bound lipids and another which is modulated by the bulk membrane lipids and is responsible for part of the inhibition of the overall (Na^+ + K^+)-ATPase activity (the major part of the inhibition of the overall reaction is due to the inhibition of the phosphatase reaction). This scheme could accomodate the phosphatase being a part of the same enzyme macromolecule as the (Na^+ + K^+)-ATPase but with a different lipid requirement.

D. Temperature Dependence

Temperature-dependence has been defined in this thesis as the temperature-activity relationship of an enzyme as displayed by an Arrhenius plot from which values for activation energies (Ea) and for the critical temperature (Tc) can be calculated. This parameter is an important factor in describing the nature of the lipid modulation of the ($Na^+ + K^+$)-ATPase and K^+-O.I.-*p*-NPPase reactions.

Whereas the specific activity of an enzyme reaction describes the number of active sites per unit of tissue, the activation energy calculated from the slope of the Arrhenius plot describes the thermodynamic efficiency of each active site (33). Although the energy required to activate an overall reaction can be calculated from the slope of an Arrhenius plot, the activation energy of a rate limiting step [and probably any other component step of the reaction sequence] cannot be deduced from this slope (161). However, Barnett and Palazzotto (18) disagree with the above statement and believe that the change in slope of a non-linear Arrhenius plot does reflect the change in activation energy of the rate-limiting step of a reaction sequence. Because the phosphorylated enzyme intermediate $E_2 \sim P$ tended to accumulate at all temperatures when K^+ was not present in the assay medium, they believed that the rate limiting step must be the breakdown of $E_2 \sim P$ to $E_2 + P_i$, i.e., the phosphatase reaction. Below 20°C however, they believed that the conversion of an $E_2 \sim K$ complex to E_1 represented the rate limiting step (determined by Post *et al.* (1972), cited by Barnett and Palazzotto (18)).

The presence or absence of a distinct inflection point in an Arrhenius plot of enzyme activity and its location provide additional information about the lipid modulation of the enzyme reaction. Van Dijck (150) found that the inflections in Arrhenius plots of transport processes corresponded to the transition temperatures of human and bovine erythrocyte membranes as measured by differential scanning calorimetry (d.s.c.). Kimelberg and Papahadjopoulos (72) similarly found a good correlation between the critical temperature of their membrane preparation from rabbit kidney outer medulla measured by d.s.c. and the inflection point of a non-linear Arrhenius plot of ($Na^+ + K^+$)-ATPase activity of this membrane preparation. Furthermore, because d.s.c. measured bulk membrane properties, they believed that the specific activity was then a more sensitive indicator of fluidity changes in the vicinity of the membrane. In other words, they were postulating the existence of a micro-environment of lipids more directly responsible for modulating ($Na^+ + K^+$)-ATPase activity, and whose phase changes were reflected by the shape of the Arrhenius plot. Because the critical temperature is characteristic for a lipid, its value could be used to identify a specific lipid. However, in a heterogeneous system of lipids and proteins which constitute a biological membrane, the phase transition is not sharp, but occurs over a wider range. The point of inflection of a non-linear Arrhenius plot would then represent a temperature within the phase transition. Therefore, identification of a specific lipid required to modulate enzyme activity could not be achieved solely by this technique, but would require more accurate methods, e.g., extraction of the lipids from the membrane followed by chromatography.

1. Lipolysis

Examination of the temperature dependence of ($Na^+ + K^+$)-ATPase and K^+-O.I.-*p*-NPPase of membrane preparations delipidated by phospholipase treatments again differentiated between the lipid requirements of these two enzyme reactions.

The lipid dependence of the overall ($Na^+ + K^+$)-ATPase seemed to be eliminated after PPLA, PPLC, or (PPLA + PPLC) treatment of a membrane preparation which had been pretreated with DOC as demonstrated by the conversion from non-linear to linear Arrhenius plots following these procedures

(Fig. 19a, 21a, 23a). When an untreated enzyme preparation was incubated with PPLC, however, the Arrhenius plot still appeared as two straight lines intersecting at about 20°C (Fig. 20a). The ratio of $Ea_I:Ea_{II}$ was increased to 0.6 from 0.4 for control preparations which indicated that the curve was approaching linearity.

In contrast to the results with $(Na^+ + K^+)$-ATPase, the temperature dependence of the partial phosphatase reaction was not altered greatly by lipase treatments as demonstrated by the non-linear Arrhenius plots of K^+-O.I.-*p*-NPPase activity which persisted despite the treatments. The Arrhenius plot appeared as two intersecting straight lines with values for Ea_I and Ea_{II} and Tc which were similar to those of control values (Fig. 19b, 21b, 23b). However, when an untreated enzyme preparation was incubated with PPLC or when a DOC-treated preparation in the presence of EDTA was the substrate, the Arrhenius plot of K^+-O.I.-*p*-NPPase activity was curvilinear and the data did not permit the calculation of distinct activation energies (Fig. 20b, 22b). Because lipase treatments did not markedly alter the temperature dependence of K^+-O.I.-*p*-NPPase activity, these results suggested that the phosphatase activity was not modulated by those lipids which were removed by DOC or lipase treatments. It was probably modulated by a microenvironment of lipids which were tightly bound to the vicinity of the active site of this enzyme reaction and which were not accessible to lipase action under our conditions. The decrease in specific activity of K^+-O.I.-*p*-NPPase after lipase treatment suggested that the number of active sites was decreased, although the efficiency of each, i.e., the activation energy of the process, was maintained as demonstrated by the similarity of the values of the activation energies that were obtained before and after lipase treatments (when the curves were such that activation energies could be calculated).

Lipolytic agents would be expected to first disrupt lipid interactions in the bulk phase of the membrane bilayer before they could gain access to the more tightly bound lipids associated with intrinsic proteins. It could then be suggested that the overall (Na^+ + K^+)-ATPase reaction depended more on the mulk membrane lipids than on the tightly bound lipids, while the partial phosphatase reaction did depend on the tightly bound lipids for its modulation, if it was lipid-dependent at all.

The removal of certain phospholipids from the membrane matrix by PPLA activity would reduce the close packing of the remaining membrane components at all temperatures. Similarly, the removal of polar head groups by PPLC could permit the elongation and greater mobility of the diglycerides remaining in the membrane, and perhaps, aggregation into droplets as described by Azhar *et al.* (16) which would also decrease the packing density. This decreased packing and increased mobility could be described as a general increase in the fluidity of the membrane at all temperatures examined, thereby abolishing the sharp phase transition seen before.

Because a single activiation energy could be calculated from a linear Arrhenius plot of (Na^+ + K^+)-ATPase activity after lipase treatment, therefore, the enzyme was adopting a single energy level over the temperature range examined in the more fluid membrane matrix. Because the value of the single activation energy now obtained was intermediate between the two values of Ea_I and Ea_{II} of the control preparations this finding indicates that the (Na^+ + K^+)-ATPase of a lipase treated preparation required less energy to function at temperatures below the previous inflection point than a control preparation, but also required more energy to function at temperatures above the previous inflection point. (This relationship held true even in the case of the (Na^+ + K^+)-ATPase of a membrane preparation treated only with PPLC and not pretreated with DOC, where the Arrhenius plot was still non-linear, but the ratio $Ea_I:Ea_{II}$ was approaching 1.0.) This result could suggest that the increased fluidity of the membrane facilitated the reaction mechanism at lower temperatures, but perhaps did not provide enough structural support to facilitate the reaction (especially the conformational change steps) at the higher temperatures, thus resulting in the observed changes in activation energies.

The curvilinear Arrhenius plot which resulted after PPLC treatment of an untreated enzyme preparation or of a DOC-treated preparation in the presence of EDTA demonstrated that the K^+-O.I.-*p*-NPPase activity did not adopt a single energy level but instead adopted different levels within the temperature range examined. The continuous change in energy levels of the phosphatase activity could have been made possible by an increased fluidity of the micro-environment of lipids about the phosphatase active site or by an increased flexibility of the complex consisting of the phosphatase active site plus the surrounding lipids within the more fluid membrane matrix resulting after the above described lipid treatments.

When EDTA was included in the incubation medium with a DOC-treated enzyme preparation prior to PPLC treatment, its chelation of Ca^{++} and other divalent ions enhanced the effectiveness of PPLC action, as described above (section A). Perhaps, the increased accessibility of PPLC to some of the lipids of the enzyme's micro-environment resulted in an enhanced mobility at the phosphatase active site. This enhanced mobility was demonstrated by a curvilinear Arrhenius plot of phosphatase activity and the absence of a distinct value for the activation energy over the temperature range examined and the absence of a distinct inflection point. Because PPLC treatment of an untreated preparation probably did not reach the lipids of the micro-environment, the curvilinear Arrhenius plot of K^+-O.I.-*p*-NPPase activity was probably due to the increased flexibility of the phosphatase-lipid complex within the more fluid membrane matrix.

It was found that "double" treatments with PPLA further disrupted the membrane matrix, since the Arrhenius plots of both $(Na^+ + K^+)$-ATPase and K^+-O.I.-*p*-NPPase activities now produced higher activation energies, although the qualitative appearance of the plots did not change. Because the activation energies were higher after a second lipolysis, the efficiency of each active site had therefore decreased with the loss of lipids, thus again confirming the lipid dependence of these reactions.

Our results using beef brain enzyme preparations agree directly with those of Wheeler (157) who found that the K^+-phosphatase activity of rabbit kidney preparations was not closely related to the phospholipid composition of the membrane preparation and did not possess as stringent lipid requirements as the overall $(Na^+ + K^+)$-ATPase reaction in their system. Our results are also compatible with those of Kimelberg and Papahadjopoulos (72) who believed that the inflection point of a non-linear Arrhenius plot of $(Na^+ + K^+)$-ATPase activity was a more sensitive indicator of the transition temperature of the membrane system and in particular of the lipids modulating the reaction. Extrapolating this belief to the K^+-O.I.-*p*-NPPase activity suggests that this activity is modulated by tightly bound lipids which are not greatly influenced by various lipase treatments and whose phase transition is demonstrated by the inflection point of biphasic Arrhenius plots.

Because the active sites of the overall $(Na^+ + K^+)$-ATPase and of the partial K^+-O.I.-*p*-NPPase are located on opposite sides of the membrane bilayer as discussed above, a comparison of the temperature dependence of these two activities again suggests that there is an asymmetric membrane distribution of the phospholipids which modulate these activities and which are substrates for PPLA and PPLC action. Such a model could accomodate the postulate that both activities occur on the same enzyme macromolecule but possess different lipid requirements as demonstrated by the nonparallel results obtained after lipolytic treatments.

2. Summary of temperature dependence

A study of the temperature-activity relationships of lipase-treated enzyme preparations appeared to support the postulate of asymmetric distribution of the membrane phospholipids across the bilayer and about the respective active sites of $(Na^+ + K^+)$-ATPase and K^+-O.I.-*p*-NPPase. The lipid requirement of the phosphatase reaction appeared to be less stringent than the lipid requirement of the overall $(Na^+ + K^+)$-ATPase reaction, and might be expressed by a group of tightly bound lipids in the close vicinity of the phosphatase active site. In this manner both activities could occur on the same enzyme macromolecule and could be modulated by different membrane lipids in different ways.

E. Conclusions

It can be recalled that our results demonstrated that phospholipase-treatment of untreated or DOC-treated beef brain microsomes lowered the specific activity of both $(Na^+ + K^+)$-ATPase as well as K^+-O.I.-*p*-NPPase, although not always in parallel, did not greatly alter the ouabain inhibition of either enzyme reaction, and altered the temperature dependence of both enzyme reactions in different ways depending on the lipolytic treatment.

Examination of the three parameters of enzyme activity, namely specific activity, ouabain inhibition, and temperature dependence of $(Na^+ + K^+)$-ATPase and K^+-O.I.-*p*-NPPase activities of phospholipase treated membrane preparations, leads to the suggestion that the lipids of the membrane are asymmetrically distributed in the bilayer and that there is a different lipid requirement for the modulation of each distinct enzyme activity. The postulate that the partial K^+-O.I.-*p*-NPPase activity is located on the same enzyme macromolecule as the overall ($Na^+ + K^+$)-ATPase activity could be retained by proposing that the lipid environment of the active sites of these enzyme reactions is different, i.e., the modulating lipids could be asymmetrically distributed in the membrane about the enzyme complex.

The lipids of the bulk phase could be more involved in modulating the overall ($Na^+ + K^+$)-ATPase reaction (especially at the level of its conformational changes), while the lipids more closely associated with the enzyme complex could be more involved in modulating the partial phosphatase reaction.

The interpretation of these results tends to suggest the existence of annular lipids about the ($Na^+ + K^+$)-ATPase which probably have a particular role in ligand binding. Such an annulus has been described for some other enzymes, including ($Ca^{++} + Mg^{++}$)-ATPase (79, 100, 101). This suggestion could be substantiated by examining the ($Na^+ + K^+$)-ATPase - containing preparations before and after various lipid treatments employing electron microscopic and/or freeze fracture techniques.

The following model is proposed to differentiate between the requirements of the enzyme for lipids of the bulk phase and for more tightly bound lipids:

Because the overall ($Na^+ + K^+$)-ATPase reaction sequence consists of component reactions which include ligand binding to the enzyme as well as conformational changes, we propose that such major molecular reorientations of the enzyme required to undergo the conformational changes of the reaction sequence would be influenced more by the lipids of the bulk phase whereas reaction steps involving the binding of ligands would involve the lipids which were more closely associated with the enzyme.

According to this model, the altered temperature dependence of the overall ($Na^+ + K^+$)-ATPase reaction is a reflection of the disruption of the lipid interactions of the bulk membrane phase by lipases. By decreasing the packing density of the phospholipids, lipase treatment would increase membrane fluidity at all temperatures and would permit the entire enzyme complex to adopt a single energy level to facilitate the conformational change steps (designated as $E_1 \sim P \rightleftarrows E_2 \sim P$ and $E_2 \longrightarrow E_1$) which depend on the bulk membrane lipids. The non-linear Arrhenius plots of K^+-O.I.-*p*-NPPase activity would then reflect modulation by the lipids more closely associated with the enzyme (at the K^+ binding sites, in particular) which were not altered by lipase treatment. Even if some of these lipids had been altered by lipase treatment, their close association with the enzyme would not permit them the same enhanced mobility after lipase treatment as the bulk phase lipids demonstrated. Therefore, the non-linear Arrhenius plots of phosphatase activity reflect the changes in energy levels which the enzyme undergoes within the temperature range examined. This model for lipid dependence is compatible with one recently proposed by Wheeler (157) and described in the Introduction to this disertation (section 6-d,p.48). They similarly concluded that the phosphatase reaction depended on tightly bound lipids, although they also considered that this particular reaction might not be lipid dependent at all!

Our original questions should now be recalled:

1. is there a requirement for a particular lipid(s) to modulate the phosphatase reaction? and
2. if so, what is the function of this lipid in the phosphatase reaction?

Although they cannot be precisely identified from the present work, the particular phospholipids required to modulate the phosphatase reaction appear to be those which are tightly bound to the enzyme and which are not easily disturbed by our various lipolytic treatments. Without doing more specific lipid analyses, a more precise answer cannot be given at present, although speculations could be made based on integrating our findings with the work of other investigators (57, 71, 72).

The function of the lipids appears to be to aid in the binding of substrates and regulating ions by providing a suitable micro-environment about the binding sites, and to assist in the conformational changes of the (Na^+ + K^+)-ATPase during the reaction sequence.

Besides being involved in the normal functioning of the enzyme, the lipids are also involved in binding its inhibitors, notably the cardiac glycosides. The study of the binding of ouabain supported the evidence that two drug binding sites might exist on this system, one of which was more lipid dependent than the other (35), and which probably is the one responsible for inhibiting the phosphatase reaction.

The pharmacological interest in the (Na^+ + K^+)-ATPase is due to its rôle as the putative cardiac glycoside receptor. Inhibition of this enzyme by cardiac glycosides is believed to be the mechanism by which the drugs demonstrate their positive inotropic action in the failing heart (73, 120, 168). Characterization or identification of the specific lipids involved in the regulation of phosphatase activity and, hence, of glycoside binding, would provide valuable information to aid in a more efficient and rational therapeutic use of these drugs in congestive heart failure.

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